PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C11D 3/386, C12N 9/18
// C12N 15/55, 9/18

A1

(11) International Publication Number: WO 94/03578

(43) International Publication Date: 17 February 1994 (17.02.94)

(21) International Application Number: PCT/EP93/01923

(22) International Filing Date: 20 July 1993 (20.07.93)

(30) Priority data:
9216387.2 31 July 1992 (31.07.92) GB

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ENZYMATIC DETERGENT COMPOSITIONS

(57) Abstract

There is provided an enzymatic detergent composition which comprises: (a) 0.1 - 50 % by weight of a surfactant system comprising (a1) 0 - 95 % by weight of one or more anionic surfactants and (a2) 5 - 100 % by weight of one or more nonionic surfactants; and (b) 10 - 20,000 LU per gram of the detergent composition of an enzyme which is capable of exhibiting a substantial lipolytic activity during the main cycle of a wash process.

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ENZYMATIC DETERGENT COMPOSITIONS

TECHNICAL FIELD

The present invention generally relates to the field of enzymatic detergent and cleaning compositions. More in particular, the invention is concerned with enzymatic detergent compositions comprising enzymes having lipolytic activity.

BACKGROUND AND PRIOR ART

Various types of enzymes are known as additives for detergent compositions. For example, detergent compositions containing proteases, cellulases, amylases, lipases and various combinations thereof have been described in the literature and several such products have appeared on the market. The present invention is concerned with detergent compositions comprising lipolytic enzymes or lipases. Such enzymes could contribute to the removal of fatty soil from fabrics by hydrolysing one or more of the ester bonds in triglycerides.

EP-A-214 761 (Novo Nordisk) discloses lipases which are derived from organisms of the species Pseudomonas cepacia, and EP-A-258 068 (Novo Nordisk) discloses lipases which are derived from organisms of the genus Thermomyces (previous name Humicola). Both patent applications also describe the use of these lipases as detergent additives.

Further examples of lipase-containing detergent compositions are provided by EP-A-205 208 and EP-A-206 390 (both Unilever), which disclose a class of lipases defined on the basis of their immunological relationships, and describe their use in detergent compositions and textile washing. The preferred lipases are those from Pseudomonas fluorescens, Pseudomonas gladioli and Chromobacter species.

EP-A-331 376 (Amano) describes lipases, their use and their production by means of recombinant DNA (rDNA) techniques, and includes an amino acid sequence of lipase from Pseudomonas cepacia. Further examples of lipase enzymes

produced by means of rDNA techniques are given in WO-A-89/09263 and EP-A-218 272 (both Gist-Brocades).

In spite of the large number of publications on lipase enzymes and their modifications, only the lipase derived from Humicola lanuginosa and produced in Aspergillus oryzae as host has so far found wide-spread application as additive for fabric washing products. It is available from Novo-Nordisk under the trade name Lipolase (TM).

In his article in Chemistry and Industry 1990, pages 183-186, Henrik Malmos notes that it is known that generally the activity of lipases during the washing process is low, and Lipolase (TM) is no exception. During the drying process, when the water content of the fabric is reduced, the enzyme regains its activity and the fatty stains are hydrolysed. During the following wash cycle the hydrolysed material is removed. This also explains why the effect of lipases is low after the first washing cycle, but significant in the following cycles. These findings are also described by Aaslyng et al. (1991), in "Mechanistic Studies of Proteases and Lipases for the Detergent Industry", J.Chem.Tech. Biotechnol. 50, 321-330.

The inventors of the present application regard it as a disadvantage of the existing detergent products which contain a lipolytic enzyme, that no significant benefit can be expected from the lipolytic enzyme when the products are used to wash fabrics which have not been in contact with the detergent product before.

Furthermore, there is a growing tendency in Europe and the United States among the users of automatic washing machines to employ tumble dryers for drying the washed fabrics. In these machines, the wet cloths are rapidly dried by contacting them with hot air. The drying process which normally may take from 6 to 48 hours at ambient temperature, can be shortened to less than one hour using a tumble dryer. As said above, the lipase enzyme regains its activity during the drying process, when the water content of the fabric is reduced. The period of optimal water content for the lipase is strongly shortened when a tumble dryer is used.

Consequently, the consumer will benefit less from the presence of conventional lipase enzymes in a detergent product, when the product is used in a wash process which includes a drying step in a tumble dryer.

It is therefore an object of the present invention to provide an enzymatic detergent composition which exhibits a substantial lipolytic activity during the main cycle of a wash process in an automatic washing machine, and which consequently will exhibit lipolytic activity when used to wash fabrics which have not been in contact with the detergent product before. It is also an object of the present invention to provide an enzymatic detergent composition which is especially suitable for use in combination with a tumble dryer.

It is a further object of the present invention to provide a process for producing such an enzyme which exhibits a substantial lipolytic activity during the main cycle of a wash process in an automatic washing machine.

We have now surprisingly found that there exist lipolytic enzymes which can be used to formulate detergent compositions which exhibit a substantial lipolytic activity during the main cycle of a wash process. Furthermore, we have found that there is a good correlation between the capability of exhibiting such a lipolytic activity during the main cycle of a wash process and their inactivation behaviour with Di-isopropyl Fluoro Phosphate (DFP). Thus, suitable lipolytic enzymes can be conveniently selected on the basis of their inactivation behaviour with DFP. In particular, cutinase enzymes of eukaryotic origin were found to be suitable enzymes exhibiting such a lipolytic effect during the main cycle of a wash process.

WO-A-88/09367 (Genencor) suggests combinations of a surfactant and a substantially pure microbial cutinase enzyme to formulate effective cleaning compositions. Disclosed are detergent compositions comprising a cutinase obtained from the (prokaryotic) Pseudomonas putida ATCC 53552. However, in the more recent European patent application EP-A-476 915 (Clorox), it is disclosed that the same enzyme - which is

then referred to as a lipase - is no more effective than other lipases in removing oil stains from fabrics, when used by conventional methods. In other words, this enzyme is not believed to exhibit an in-the-wash effect.

WO-A-90/09446 (Plant Genetics Systems) describes the cloning and production of a eukaryotic cutinase from Fusarium solani pisi in E. coli, and mentions inter alia that this cutinase could be used to produce cleaning agents such as laundry detergents and other specialized fat dissolving preparations such as cosmetic compositions and shampoos. Specific enzymatic detergent compositions are not disclosed or suggested, so that the skilled man would find no incentive to select this particular enzyme to formulate detergent compositions for fabric washing.

DEFINITION OF THE INVENTION

According to a first aspect of the invention, there is provided an enzymatic detergent composition comprising:

(a) 0.1 - 50 % by weight of an active system which comprises

(a1) 0 - 95 % by weight of one or more anionic surfactants and

(a2) 5 - 100 % by weight of one or more nonionic surfactants; and

(b) 10 - 20,000 LU per gram of the detergent composition of an enzyme which is capable of exhibiting a substantial lipolytic activity during the main cycle of a wash process.

According to a second aspect of the invention, there is provided a process for identifying and selecting an enzyme which exhibits a substantial lipolytic activity during the main cycle of a wash process in an automatic washing machine on the basis of its inactivation behaviour with Di-isopropyl Fluoro Phosphate (DFP).

According to a further aspect of the invention, there is provided a process for producing such an enzyme.

DESCRIPTION OF THE INVENTION

(a) The surfactant system

The invention in one of its aspects provides an enzymatic detergent composition comprising from 0.1 - 50 % by weight of an active system, which in turn comprises 0 - 95 % by weight of one or more anionic surfactants and 5 - 100 % by weight of one or more nonionic surfactants. The surfactant system may additionally contain amphoteric or zwitterionic detergent compounds, but this in not normally desired owing to their relatively high cost.

In general, the nonionic and anionic surfactants of the surfactant system may be chosen from the surfactants described "Surface Active Agents" Vol. 1, by Schwartz & Perry, Interscience 1949, Vol. 2 by Schwartz, Perry & Berch, Interscience 1958, in the current edition of "McCutcheon's Emulsifiers and Detergents" published by Manufacturing Confectioners Company or in "Tenside-Taschenbuch", H. Stache, 2nd Edn., Carl Hauser Verlag, 1981.

Suitable nonionic detergent compounds which may be used include, in particular, the reaction products of compounds having a hydrophobic group and a reactive hydrogen atom, for example, aliphatic alcohols, acids, amides or alkyl phenols with alkylene oxides, especially ethylene oxide either alone or with propylene oxide. Specific nonionic detergent compounds are C_6-C_{22} alkyl phenol-ethylene oxide condensates, generally 5 to 25 EO, i.e. 5 to 25 units of ethylene oxide per molecule, and the condensation products of aliphatic C_8-C_{18} primary or secondary linear or branched alcohols with ethylene oxide, generally 5 to 40 EO.

Suitable anionic detergent compounds which may be used are usually water-soluble alkali metal salts of organic sulphates and sulphonates having alkyl radicals containing from about 8 to about 22 carbon atoms, the term alkyl being used to include the alkyl portion of higher acyl radicals. Examples of suitable synthetic anionic detergent compounds are sodium and potassium alkyl sulphates, especially those obtained by sulphating higher C_8-C_{18} alcohols, produced for example from tallow or coconut oil, sodium and potassium

alkyl C_9-C_{20} benzene sulphonates, particularly sodium linear secondary alkyl C_{10}-C_{15} benzene sulphonates; and sodium alkyl glyceryl ether sulphates, especially those ethers of the higher alcohols derived from tallow or coconut oil and synthetic alcohols derived from petroleum. The preferred anionic detergent compounds are sodium C_{11}-C_{15} alkyl benzene sulphonates and sodium C_{12}-C_{18} alkyl sulphates.

Also applicable are surfactants such as those described in EP-A-328 177 (Unilever), which show resistance to salting-out, the alkyl polyglycoside surfactants described in EP-A-070 074, and alkyl monoglycosides.

Preferred surfactant systems are mixtures of anionic with nonionic detergent active materials, in particular the groups and examples of anionic and nonionic surfactants pointed out in EP-A-346 995 (Unilever). Especially preferred is surfactant system which is a mixture of an alkali metal salt of a C_{16}-C_{18} primary alcohol sulphate together with a C_{12}-C_{15} primary alcohol 3-7 EO ethoxylate.

The nonionic detergent is preferably present in amounts greater than 10%, e.g. 25-90% by weight of the surfactant system. Anionic surfactants can be present for example in amounts in the range from about 5% to about 40% by weight of the surfactant system.

(b) The enzyme

The enzymatic detergent compositions of the invention further comprise 10 - 20,000 LU per gram, and preferably 50 - 2,000 LU per gram of the detergent composition, of an enzyme which is capable of exhibiting a substantial lipolytic activity during the main cycle of a wash process. In this specification LU or lipase units are defined as they are in EP-A-258 068 (Novo Nordisk).

By a substantial lipolytic activity during the main cycle of a wash process, or in-the-wash effect, it is meant that a detergent composition containing the enzyme is capable of removing a significant amount of oily soil from a soiled fabric in a single wash process in a European type of automatic washing machine, using normal washing conditions as

far as concentration, water hardness, temperature, are concerned. It should be born in mind that under the same conditions, the conventional commercially available lipolytic enzyme Lipolase (TM) ex Novo Nordisk does not appear to have any significant in-the-wash effect on oily soil.

The in-the-wash effect of an enzyme on oily soil can be assessed using the following assay. New polyester/cotton test cloths having a cotton content of 33% are prewashed using an enzyme-free detergent product such as the one given below, and are subsequently thoroughly rinsed. Such unsoiled cloths are then soiled with olive oil or another suitable, hydrolysable oily stain. Each tests cloth (weighing approximately 1 g) is incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The wash liquor contains the detergent product given below at a dosage of 1 g per litre. The bottles are agitated for 30 minutes in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The enzyme having lipolytic activity is preadded to the wash liquor at 3 LU/ml. The control does not contain any enzyme. The washing powder has the following composition (in % by weight):

Ethoxylated alcohol nonionic surfactant	9.5
Sodium sulphate	38.6
Sodium carbonate	40.4
Sodium silicate ($Na_2O:Si_2O$ = 2.4)	7.3
Water	4.2

As nonionic surfactant we used C_{12}-C_{15} ethoxylated alcohol 10.5-13 EO, but it was found that the nature of the ethoxylated alcohol nonionic can vary within wide limits.

After washing, the cloths are thoroughly rinsed with cold water and dried in a tumble dryer with cold air, and the amount of residual fat is assessed. This can be done in several ways. The common method is to extract the testcloth with petroleum ether in a Soxhlet extraction apparatus, distilling off the solvent and determining the percentage residual fatty material as a fraction of the initial amount of fat on the cloth by weighing.

According to a second, more sensitive method, brominated olive oil is used to soil the test cloths (Richards, S., Morris, M.A. and Arklay, T.H. (1968), Textile Research Journal 38, 105-107). Each test cloth is then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. A series of bottles is then agitated in a washing machine filled with water and using a normal 30°C main wash programme. After the main wash, the test cloths are carefully rinsed in cold water during 5 seconds. Immediately after the rinse, the test cloths dried in a dryer with cold air. After drying the amount of residual fat can be determined by measuring the bromine content of the cloth by means of X-ray fluorescence spectrometry. The fat removal can be determined as a percentage of the amount which was initially present on the test cloth, as follows:

$$\% \text{ Soil removal} = \frac{\text{Bromine}_{bw} - \text{Bromine}_{aw}}{\text{Bromine}_{bw}} * 100\ \%$$

wherein: Bromine_{bw} denotes the percentage bromine on the cloth before the wash and Bromine_{aw} the percentage bromine after the wash.

A further method of assessing the enzymatic activity is by measuring the reflectance at 460 nm according to standard techniques.

According to the invention, the detergent composition comprises an enzyme which is capable of removing at least 5% more, preferably at least 10% more oily soil, on the basis of the initial amount of oily soil, than the same detergent composition without the enzyme, in the assay as herein described.

Another way of defining the enzyme activity is by relating it to the activity of the commercially available Lipolase (TM), a lipase which can be obtained from Novo/Nordisk. According to the invention, the ratio of enzymatic oily soil removal by the enzyme to the enzymatic oily soil removal by Lipolase (TM) in the assay as herein described is at least 3, preferably at least 5. By enzymatic soil removal is meant the soil removal which can be

attributed to the presence of the enzyme, i.e. by subtracting as background the soil removal which is observed in the absence of the enzyme.

Now that it has been disclosed to him by the present application that is possible to formulate detergent compositions having a substantial in-the-wash effect, it will be clear to the skilled man how to select a suitable enzyme for use in the present invention. However, we have found a very convenient method for selecting suitable lipolytic enzymes for the compositions of the invention, which method is based on our finding that the in-the wash effect of lipolytic enzymes is closely correlated to their inactivation behaviour with Di-isopropyl Fluoro Phosphate (DFP). It is generally known that this compound reacts readily with the active site serine residue in lipolytic enzymes which thereby lose their enzymatic activity. We have found that lipolytic enzymes which are readily inactivated by DFP, i.e. which possess an accessible active site serine residue, generally have good in-the-wash effect. On the other hand, lipolytic enzymes which are more slowly inactivated by DFP, i.e. which possess an inaccessible active site serine residue, generally have no or a very poor in-the-wash effect. Lipolytic enzymes which exhibit a residual activity of less than 50%, preferably less than 40%, after a 10 minute incubation with DFP at pH 10 at room temperature, were found to have a good in-the-wash effect. Even better in-the-wash effects were found with lipolytic enzymes having a residual activity of less than 25% and the best effects were found with lipolytic enzymes having a residual activity of less than 15%. The details of the DPF-inactivation assay can be found in the Examples.

Suitable enzymes for the compositions of the invention can be found in the enzyme classes of the esterases and lipases, (EC 3.1.1.*, wherein the asterisk denotes any number).

A much preferred type of enzyme exhibiting an in-the-wash effect according to the invention is a eukaryotic cutinase. Cutinases are a sub-class of enzymes (EC 3.1.1.50),

the wax ester hydrolases. These enzymes are capable of degrading cutin, a network of esterified long-chain fatty acids and fatty alcohols which occurs in plants as a protective coating on leaves and stems. In addition, they possess some lipolytic activity, i.e. they are capable of hydrolysing triglycerides. Thus they can be regarded as a special kind of lipases.

A characteristic feature of lipases is that they exhibit interfacial activation. This means that the enzyme activity is much higher on a substrate which has formed interfaces or micelles, than on fully dissolved substrate. Interface activation is reflected in a sudden increase in lipolytic activity when the substrate concentration is raised above the critical micel concentration (CMC) of the substrate, and interfaces are formed. Experimentally this phenomenon can be observed as a discontinuity in the graph of enzyme activity versus substrate concentration. Contrary to lipases, however, cutinases do not exhibit any substantial interfacial activation.

Because of this characteristic feature, i.e. the absence of interfacial activation, we define for the purpose of this patent application Cutinases as lipolytic enzymes which exhibit substantially no interfacial activation. Cutinases therefor differ from classical lipases in that they do not possess a helical lid covering the catalytic binding site.

Because of their fat degrading properties, cutinases in general have been proposed as ingredients for enzymatic detergent compositions. For example, WO-A-88/09367 (Genencor) suggests combinations of a surfactant and a substantially pure microbial cutinase enzyme to formulate effective cleaning compositions. Disclosed are detergent compositions comprising a (prokayotic) cutinase obtained from the Gram negative bacterium Pseudomonas putida ATCC 53552. However, as mentioned above, in the more recent European patent application EP-A-476 915 (Clorox), it is disclosed that the same enzyme - which is then referred to as a

lipase - is no more effective than other lipases in removing oil stains from fabrics, when used by conventional methods.

The cutinase gene from Fusarium solani pisi has been cloned and sequenced (Ettinger et al., (1987) Biochemistry 26, 7883-7892). WO-A-90/09446 (Plant Genetics Systems) describes the cloning and production of this gene in E. coli. The cutinase can efficiently catalyse the hydrolysis and the synthesis of esters in aqueous and non-aqueous media, both in the absence and the presence of and interface between the cutinase and the substrate. On the basis of its general stability, it is suggested that this cutinase could be used to produce cleaning agents such as laundry detergents and other specialized fat dissolving preparations such as cosmetic compositions and shampoos. A way to produce the enzyme in an economic feasible way is not disclosed, neither are specific enzymatic detergent compositions containing the cutinase.

Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. The Cutinase to be used in the present invention is chosen from the group of eukaryotic Cutinases. Such eukaryotic Cutinases can be obtained from various sources, such as plants (e.g. pollen), or fungi.

The group of (eukaryotic) fungal Cutinases appears to comprise two families with different specificities, leaf-specificity and stem-specificity. Cutinases with leaf-specificity tend to have an acidic or neutral pH-optimum, whereas Cutinases with stem-specificity tend to have an alkaline pH-optimum. Cutinases having an alkaline pH-optimum are more suitable for use in alkaline built detergent compositions such as heavy duty fabric washing powders and liquids. Cutinase having an acidic to neutral pH-optimum are more suitable for light duty products or rinse conditioners, but also for industrial cleaning products.

In the following Table I, four different stem-specific Cutinases are listed, together with their pH-optima.

TABLE I

Examples of cutinases with stem-specificity	pH-optimum
Fusarium solani pisi	9
Fusarium roseum culmorum	10
Rhizoctonia solani	8.5
Alternaria brassicicola (PNBase I)	9

Especially preferred in the present invention are Cutinases which can be derived from wild type Fusarium solani pisi (Ettinger et al., 1987). When used in suitable detergent compositions, this Cutinase exhibits clear "in-the-wash" effects.

Also preferred for the present invention are Cutinases having a high degree of homology of their amino acid sequence to the Cutinase from Fusarium solani pisi. Examples are the Cutinases from Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea.

Not suitable for the present invention is the Humicola lanuginosa lipase which is described in EP-A-305 216 (Novo Nordisk), and which is commercially available as Lipolase (TM). However, it is conceivable that this enzyme can be modified by means of rDNA techniques in such way that it also exhibits a substantial lipolytic activity during the main cycle of a wash process. Such modifications will affect the structure of the lipase enzyme. It requires therefor some experimentation to find the balance between the inevitable distortion of the conformation of the enzyme and the benefit in enzyme activity. Generally, modifications are preferred which do not affect the charge around the active site too much.

The lipolytic enzyme of the present invention can usefully be added to the detergent composition in any suitable form, i.e. the form of a granular composition, a slurry of the enzyme, or with carrier material (e.g. as in EP-A-258 068 and the Savinase (TM) and Lipolase (TM) products of Novo Nordisk). A good way of adding the enzyme to a liquid detergent product is in the form of a slurry containing 0.5 to 50 % by weight of the enzyme in a ethoxylated alcohol

nonionic surfactant, such as described in EP-A-450 702 (Unilever).

The enzyme to be used in the detergent compositions according to the invention can be produced by cloning the gene for the enzyme into a suitable production organism, such as Bacilli, or Pseudomonaceae, yeasts, such as Saccharomyces, Kluyveromyces, Hansenula or Pichia, or fungi like Aspergillus.

Naturally occurring Cutinase producing micro-organisms are usually plant pathogens and these micro-organisms are not very suitable to act as host cell for Cutinases genes. Consequently, the genes coding for (pro) Cutinases were integrated in rDNA vectors that can be transferred into the preferred host micro-organism for rDNA technology. For this purpose, rDNA vectors essentially similar to the rDNA vector described in WO-A-90/09446 can be used.

To improve the yield of the fermentation process a gene coding for a Cutinase should be transferred into micro-organisms that can growth fast on cheap medium and are capable to synthesize and secrete large amounts of the enzyme. Such suitable rDNA modified (host micro-organisms) according to the present invention are bacteria, among others, Bacilli, Corynebacteria, Staphylococci and Streptomyces, or lower eukaryotes such as Saccharomyces cerevisiae and related species, Kluyveromyces marxianus and related species, Hansenula polymorpha and related species, and species of the genus Aspergillus. Preferred host micro-organisms are the lower eukaryotes, because these micro-organisms are producing and secreting enzymes very well in fermentation processes and are able to glycolysate the Cutinase molecule. Glycosylation can contribute to the stability of the Cutinase in detergent systems.

The invention also provides genetic material derived from the introduction of eukaryotic Cutinase genes, e.g. the gene from Fusarium solani pisi, into cloning rDNA vectors, and the use of these to transform new host cells and to express the genes of the Cutinases in the new host cells.

Also provided by the invention are polynucleotides made or modified by rDNA technique, which encode such Cutinase, rDNA vectors containing such polynucleotides, and rDNA modified micro-organisms containing such polynucleotides and/or such rDNA vectors. The invention also provides corresponding polynucleotides encoding the eukaryotic Cutinases, e.g. a polynucleotide having a base sequence that encodes a mature Cutinase, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences coding for the prepro- or pro-sequence of this Cutinase directly upstream of the nucleotide sequences coding for the mature Cutinase.

In such a polynucleotide, the Cutinase-encoding nucleotide sequence derived from the organism of origin can be modified in such a way that at least one codon, and preferably as many codons as possible, are made the subject of 'silent' mutations to form codons encoding equivalent amino acid residues and being codons preferred by a new host, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

Upstream of the nucleotide sequences coding for the pro-or mature Cutinases, there can be located a nucleotide sequence that codes for a signal or secretion sequence suitable for the chosen host. Thus an embodiment of the present invention relates to a rDNA vector into which a nucleotide sequence coding for a Cutinase or a precursor thereof has been inserted.

The nucleotide sequence can be derived for example from:

(a) a naturally occurring nucleotide sequence (e.g. encoding the original amino acid sequence of the propre- or pro-cutinase produced by Fusarium solani pisi);

(b) chemically synthesized nucleotide sequences consisting of codons that are preferred by the new host and a nucleotide sequence resulting in stable messenger RNA in the new host, still encoding the original amino acid sequence;

(c) genetically engineered nucleotide sequences derived from one of the nucleotide sequences mentioned in preceding

paragraphs a or b coding for a Fusarium solani pisi Cutinase with a different amino acid sequence but having superior stability and/or activity in detergent systems.

Summarizing, rDNA vectors able to direct the expression of the nucleotide sequence encoding a Cutinase gene as described above in one of the preferred hosts preferably comprise the following components:

(a) Double-stranded (ds) DNA coding for mature Cutinase or precutinase or a corresponding precutinase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell). In cases where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front. The translated part of the gene should always end with an appropriate stop codon;

(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Cutinase (component (a));

(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Cutinase (component (a);

(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,

(d2) an origin of replication suitable for the selected host;

(e1) Optionally a (auxotrophic) selection marker. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter;

(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the Cutinase in the host selected.

Such a rDNA vector can also carry, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the cutinase. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter region.

The invention also provides a process for producing a lipolytic enzyme capable of exhibiting an in-the-wash

effect, which comprises the steps of fermentatively cultivating an artificially modified micro-organism containing a gene made by rDNA technique which encodes a lipolytic enzyme having in-the-wash activity, making a preparation of the enzyme by separating the enzyme produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the enzyme either from said broth or from said cells by physical or chemical concentration or purification methods. Such a fermentation can either be a normal batch fermentation, fed-batch fermentation or continuous fermentation. The selection of a process to be used depends on the host strain and the preferred down stream processing method (known per se).

Preferably conditions are chosen such that the enzyme is secreted by the micro-organism into the fermentation broth, the enzyme being recovered from the broth after removal of the cells either by filtration or centrifugation. Optionally the enzyme can then be concentrated and purified to a desired extent.

The fermentation processes in themselves apart from the special nature of the micro-organisms can be based on known fermentation techniques and commonly used fermentation and down stream processing equipment.

In a further aspect, the invention provides artificially modified micro-organisms containing a gene for an enzyme having lipolytic activity as herein defined and able to produce the enzyme encoded by said gene.

The invention further provides recombinant DNA vectors carrying nucleotide sequences coding for lipolytic enzymes having in-the-wash activity as herein described.

(c) Other ingredients.

The enzymatic detergent composition of the present invention may further contain from 5 - 60, preferably from 20 - 50% by weight of a detergency builder. This detergency builder may be any material capable of reducing the level of

free calcium ions in the wash liquor and will preferably provide the composition with other beneficial properties such as the generation of an alkaline pH, the suspension of soil removed from the fabric and the suspension of the fabric-softening clay material.

Examples of detergency builders include precipitating builders such as the alkali metal carbonates, bicarbonates, orthophosphates, sequestering builders such as the alkali metal tripolyphosphates or nitrilotriacetates, or ion exchange builders such as the amorphous alkali metal aluminosilicates or the zeolites.

It was found to be especially favourable for the lipolytic activity of the detergent compositions of the present invention if they contained a builder material such that the free calcium concentration is reduced to less than 1 mM.

The enzymatic detergent compositions of present invention may also comprise, in further embodiments, combinations of the enzymes and other constituents normally used in detergent systems, including additives for detergent compositions. Such other components can be any of many known kinds, for example as described in GB-A-1 372 034 (Unilever), US-A-3 950 277, US-A-4 011 169, EP-A-179 533 (Procter & Gamble), EP-A-205 208 and EP-A-206 390 (Unilever), JP-A-63-078000 (1988), and Research Disclosure 29056 of June 1988, together with each of the several specifications mentioned therein. The formulation of detergent compositions according to the invention can be also illustrated by reference to the Examples D1 to D14 of EP-A-407 225 (Unilever).

Special advantage may be gained in such detergent compositions wherein a proteolytic enzyme or protease is also present. EP-A-271 154 (Unilever) describes a number of suitable proteases having a pI of lower than 10. Proteases for use together with lipases can in certain circumstances include subtilisins of for example BPN' type or of many of the types of subtilisin disclosed in the literature, some of which have already been proposed for detergents use, e.g. mutant proteases as described in for example EP-A-130 756 or

EP-A-251 446 (both Genentech), US-A-4 760 025 (Genencor), EP-A-214 435 (Henkel), WO-A-87/04661 (Amgen), WO-A-87/05050 (Genex), Thomas et al. (1986) in Nature 5, 316, and 5, 375-376 and in J.Mol.Biol. (1987) 193, 803-813, Russel et al. (1987) in Nature 328, 496-500, and others.

The invention will now be further illustrated in the following Examples. In the accompanying drawings is:

Figure 1A.

Nucleotide sequence of cassette 1 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligonucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Figure 1B.

Nucleotide sequence of cassette 2 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligonucleotides. Oligonucleotide transitions are indicated in the cassette sequence.

Figure 1C.

Nucleotide sequence of cassette 3 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligonucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Figure 1D.

Nucleotide sequence of the synthetic cutinase gene encoding Fusarium solani pisi pre-pro-cutinase. The cutinase pre-sequence, pro-sequence and mature sequence are indicated. Also the sites used for cloning and the oligonucleotide transitions are indicated. Lower case letters refer to nucleotide positions outside the open reading frame.

Figure 2.

Nucleotide sequence of a synthetic DNA fragment for linking the Fusarium solani pisi pro-cutinase encoding sequence to a sequence encoding a derivative of the E. coli phoA pre-sequence. The ribosome binding site (RBS) and the restriction enzyme sites used for cloning are indicated. Also the amino

acid sequences of the encoded phoA signal sequence and part of the cutinase gene are indicated using the one-letter code.

Figure 3.

Nucleotide sequence of cassette 8, a SacI-BclI fragment which encodes the fusion point of the coding sequences for the invertase pre-sequence and mature Fusarium solani pisi cutinase.

Figure 4.

Plasmid pUR2741 obtained by deletion of a 0.2 kb SalI-NruI from pUR2740, is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Figure 5.

Plasmid pUR7219 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with the region encoding the mature Fusarium solani pisi cutinase under the control of the yeast gal7 promoter.

Figure 6.

Plasmid pUR2740 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Figure 7.

Nucleotide sequence of cassettes 5, 6 and 7, comprising different types of connections of the coding sequences of the exlA pre-sequence and mature Fusarium solani pisi cutinase.

Figure 8.

Plasmid pAW14B obtained by insertion of a 5.3 kb SalI fragment of Aspergillus niger var. awamori genomic DNA in the SalI site of pUC19.

Figure 9.
Plasmid pUR7280 obtained by displacing the BspHI-AflII fragment comprising the exlA open reading frame in pAW14B with a BspHI-AflII fragment comprising the Fusarium solani pisi pre-pro-cutinase coding sequence. Thus, plasmid pUR7280 comprises the Fusarium solani pisi pre-pro-cutinase gene under the control of the A. niger var. awamori promoter and terminator.

Figure 10.
Plasmid pUR7281 obtained by introduction of both the A. nidulans amdS and A. niger var. awamori pyrG selection markers in pUR7280.

Figure 11.
Correlation between Residual Activity after DFP-inactivation and Percentage Soil Removal for various lipolytic enzymes.
The following abbreviations are used:

Lipolase (TM, ex Novo Nordisk)	lip
Fusarium solani pisi cutinase (Example 2)	CT
Candida cylindracea lipase (ex Sigma)	Candida
Chromobacterium viscosum lipase (ex Sigma)	Chrom
Porcine pancreas lipase (ex Sigma)	Pancr
Genencor lipase (variant of Pseudomonas putida ATCC 53552)	Gen
AKG lipase 30B (ex Amano)	AKG
SDL 195 lipase (ex Showa Denko)	SDL
Ps. pseudoalcaligines CBS 473.85 lipase	Ps.Alk

Figure 12.
A comparison of the lipolytic activity of cutinase from Fusarium solani pisi and Lipolase (TM).

Figure 13.
Single-wash effect of Fusarium solani pisi cutinase and Lipolase (TM) on different types of soiling.

Figure 14A
The effects of cutinase from Fusarium solani pisi and Lipolase (TM) at an enzyme level of 1 LU/ml, measured after a number of wash cycles,
Figure 14B idem, at enzyme level of 3LU/ml,
Figure 14C idem, at enzyme level of 5LU/ml,

Figure 14D idem, at enzyme level of 10LU/ml.

Figure 15

Multi-cycle wash test with Lipolase (TM), Pseudomonas gladioli lipase and Fusarium solani pisi cutinase in PAS/Nonionic formulation.

Figure 16

Multi-cycle wash test with Lipolase (TM), Pseudomonas gladioli lipase and Fusarium solani pisi cutinase in another PAS/Nonionic formulation.

Figure 17

Idem as Figure 16, but with resoil after each cycle.

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EXAMPLE 1

Construction of a synthetic gene encoding Fusarium solani pisi pre-pro-cutinase.

A synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was constructed essentially according to the method described in EP-A-407 225 (Unilever). Based on published nucleotide sequences of Fusarium solani pisi genes (Soliday et al. (1984) and WO-A-90/09446, Plant Genetic Systems), a completely synthetic DNA fragment was designed which comprises a region encoding the Fusarium solani pisi pre-pro-cutinase polypeptide. Compared to the nucleotide sequence of the original Fusarium solani pisi gene, this synthetic cutinase gene comprises several nucleotide changes

through which restriction enzyme recognition sites were introduced at convenient positions within the gene without affecting the encoded amino acid sequence. The nucleotide sequence of the entire synthetic cutinase gene is presented in Fig. 1D.

Construction of the synthetic cutinase gene was performed by assembly of three separate cassettes starting from synthetic DNA oligonucleotides. Each synthetic DNA cassette is equipped with an EcoRI site at the start and a HindIII site at the end. Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. For the construction of each of the cassettes the procedure outlined below was followed. Equimolar amounts (50 pmol) of the oligonucleotides constituting a given cassette were mixed, phosphorylated at their 5'-end, annealed and ligated according to standard techniques. The resulting mixture of double stranded DNA molecules was cut with EcoRI and HindIII, size-fractionated by agarose gel electrophoresis and recovered from the gel by electro-elution. The resulting synthetic DNA cassette was ligated with the 2.7 kb EcoRI-HindIII fragment of pUC9 and transformed to Escherichia coli. The EcoRI-HindIII insert of a number of clones was completely sequenced in both directions using suitable oligonucleotide primers to verify the sequence of the synthetic cassettes. Using this procedure pUR7207 (comprising cassette 1, Fig. 1A), pUR7208 (comprising cassette 2, Fig. 1B) and pUR7209 (comprising cassette 3, Fig. 1C) were constructed. Finally, the synthetic cutinase gene was assembled by combining the 2.9 kb EcoRI-ApaI fragment of pUR7207 with the 0.2 kb ApaI-NheI fragment of pUR7208 and the 0.3 kb NheI-HindIII fragment of pUR7209, yielding pUR7210. This plasmid comprises an open reading frame encoding the complete pre-pro-cutinase of Fusarium solani pisi (Fig. 1D).

EXAMPLE 2

Expression of Fusarium solani pisi (pro)cutinase in Escherichia coli.

With the synthetic cutinase gene an expression vector for E. coli was constructed which is functionally similar to the one described in WO-A-90/09446 (Plant Genetic Systems). A construct was designed in which the part of the synthetic gene encoding Fusarium solani pisi pro-cutinase is preceded by proper E. coli expression signals, i.e. (i) an inducible promoter, (ii) a ribosome binding site and (iii) a signal sequence which provides a translational initiation codon and provides information required for the export of the pro-cutinase across the cytoplasmic membrane.

A synthetic linker was designed (see Fig. 2) for fusion of a derivative of the E. coli phoA signal sequence (Michaelis et al., 1983) to the pro-sequence of the synthetic cutinase gene. To optimize cleavage of the signal peptide and secretion of pro-cutinase, the nucleotide sequence of this linker was such that the three C-terminal amino acid residues of the phoA signal sequence (Thr-Lys-Ala) were changed into Ala-Asn-Ala and the N-terminal amino acid residu of the cutinase pro-sequence (Leu 1, see Fig. 1D) was changed into Ala. This construction ensures secretion of cutinase into the periplasmatic space (see WO-A-90/09446, Plant Genetic Systems).

To obtain such a construct, the 69 bp EcoRI-SpeI fragment comprising the cutinase pre-sequence and part of the pro-sequence was removed from pUR7210 and replaced with the synthetic DNA linker sequence (EcoRI-SpeI fragment) providing the derivative of the E. coli phoA pre-sequence and the alterated N-terminal amino acid residu of the cutinase pro-sequence (Fig. 2). The resulting plasmid was named pUR7250 and was used for the isolation of a 0.7 kb BamHI-HindIII fragment comprising a ribosome binding site and the pro-cutinase encoding region fused to the phoA signal sequence encoding region. This fragment was ligated with the 8.9 kb BamHI-HindIII fragment of pMMB67EH (Fürste et al., 1986) to yield pUR7220. In this plasmid the synthetic gene encoding pro-cutinase is fused to the altered version of the phoA signal sequence and placed under the control of the inducible tac-promoter.

E. coli strain WK6 harboring pUR7220 was grown in 2 litre shakeflasks containing 0.5 litre IXTB medium (Tartof and Hobbs, 1988) consisting of:

0.017 M KH_2PO_4

0.017 M K_2HPO_4

12 g/l Bacto-tryptone

24 g/l Bacto-yeast extract

0.4 % glycerol (v/v)

Cultures were grown overnight at 25°C - 30°C in the presence of 100 µg/ml ampicillin under vigorous shaking (150 rpm) to an OD at 610 nm of 10-12. Then IPTG (isopropyl-ß-D-thiogalactopyranoside) was added to a final concentration of 10 µM and incubation continued for another 12-16 hours. When no further significant increase in the amount of produced lipolytic activity could be observed, as judged by the analysis of samples withdrawn from the cultures, the cells were harvested by centrifugation and resuspended in the original culture volume of buffer containing 20% sucrose at 0°C. The cells were collected by centrifugation and resuspended in the original culture volume of icecold water causing lysis of the cells through osmotic shock. Cell debris was removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid, left overnight at 4°C and the resulting precipitate was removed. A better than 75% pure cutinase preparation essentially free of endogenous lipases was obtained at this stage by means of ultra-filtration and freeze drying of the cell free extract. Alternatively, cutinase could be purified to homogeneity (i.e. better than 95% pure) by loading the acidified cell free extract onto SP-sephadex, eluting the enzyme with buffer at pH 8.0, passage of the concentrated alkaline solution through a suitable volume of DEAE-cellulose (Whatman DE-52) and direct application of the DEAE flow-through to a Q-sepharose HP (Pharmacia) column. Elution with a salt gradient yielded a homogenous cutinase preparation with a typical overall yield of better than 75%.

EXAMPLE 3

Expression of Fusarium solani pisi cutinase in Saccharomyces cerevisiae.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Saccharomyces cerevisiae an expression vector was constructed in which a synthetic gene encoding the mature cutinase is preceded by the pre-sequence of S. cerevisiae invertase (Taussig and Carlsson, 1983) and the strong, inducible gal7 promoter (Nogi and Fukasawa, 1983). To prepare the synthetic cutinase gene for such a fusion, an adaptor fragment was synthetized in which the coding sequence for the invertase pre-sequence is fused to the sequence encoding the N-terminus of mature cutinase. This fragment was assembled as an EcoRI-HindIII cassette in pUC9 essentially as described in Example 1 (cassette 8, see Fig. 3), yielding pUR7217. Plasmids pUR7210 and pUR7217 were transformed to E. coli JM110 (a strain lacking the dam methylase activity) and the 2.8 kb BclI-HindIII fragment of pUR7217 was ligated with the 0.6 kb BclI-HindIII fragment of pUR7210, yielding pUR7218 in which the nucleotide sequence coding for the mature cutinase polypeptide is fused with part of the S. cerevisiae invertase pre-sequence coding region.

The expression vector pUR2741 (see Fig. 4) was derived from pUR2740 (Verbakel, 1991, see Fig. 6) by isolation of the 8.9 kb NruI-SalI fragment of pUR2740, filling in the SalI protruding end with Klenow polymerase, and recircularization of the fragment. The 7.3 kb SacI-HindIII fragment of pUR2741 was ligated with the 0.7 kb SacI-HindIII fragment of pUR7218, yielding pUR7219 (see Fig. 5). Optionally, a S. cerevisiae polII terminator can be placed behind the cutinase gene, in the HindIII site, which turned out not to be essential for efficient expression of the cutinase gene. The E. coli-S. cerevisiae shuttle plasmid pUR7219 contains a origin for replication in S. cerevisiae strains harboring the 2μ plasmid (cir$^+$ strains), a promoter-deficient version of the S. cerevisiae Leu2 gene permitting selection of high copy number transformants in S. cerevisiae leu2$^-$ strains, and the synthetic gene encoding the mature

part of Fusarium solani pisi cutinase operably linked to the S. cerevisiae invertase pre-sequence under the regulation of the strong, inducible S. cerevisiae gal7 promoter.

S. cerevisiae strain SU50 (a, cir^0, leu2, his4, can1), which is identical to strain YT6-2-1L (Erhart and Hollenberg, 1981), was co-transformed with an equimolar mixture of the 2μ S. cerevisiae plasmid and pUR7219 using a standard protocol for electroporation of yeast cells. Transformants were selected for leucine prototrophy and total DNA was isolated from a number of transformants. All transformants appeared to contain both the 2μ plasmid and pUR7219, exemplifying that the promoter-deficient version of the leu2 gene contained on pUR7219 can only functionally complement leu2 deficient strains when present in high copy numbers due to the simultaneous presence of the 2μ yeast plasmid. One of the transformants was cured for the pUR7219 plasmid by cultivation on complete medium for more than 40 generations followed by replica-plating on selective and complete solid media, yielding S. cerevisiae strain SU51 (a, cir$^+$, leu2, his4, can1).

S. cerevisiae strain SU51 harboring pUR7219 was grown in 1 litre shakeflasks containing 0.2 litre MM medium consisting of:

- yeast nitrogen base (YNB) without amino acids	6.7	g/l
- histidine	20	mg/l
- glucose	20	g/l

Cultures were grown overnight at 30°C under vigorous shaking (150 rpm) to an OD at 610 nm of 2-4. Cells were collected by centrifugation and resuspended in 1 litre of YPGAL medium consisting of:

- yeast extract	10	g/l
- bacto peptone	20	g/l
- galactose	50	g/l

in 2 litre shake flasks and incubation continued for another 12-16 hours. At regular intervals samples were withdrawn from the culture and centrifugated to remove biomass. The supernatant was analyzed for cutinase activity by a titrimatic assay using olive oil as a substrate. For each

sample between 100 and 200 µl of filtrate was added to a stirred mixture of 5.0 ml lipase substrate (Sigma, containing olive oil as a substrate for the lipase) and 25.0 ml of buffer (5 mM Tris-HCl pH 9.0, 40 mM NaCl, 20 mM $CaCl_2$). The assay was carried out at 30°C and the release of fatty acids was measured by automated titration with 0.05 M NaOH to pH 9.0 using a Mettler DL25 titrator. A curve of the amount of titrant against time was obtained. The amount of lipase activity contained in the sample was calculated from the maximum slope of this curve. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of fatty acid from olive oil in one minute under the conditions specified above. Such determinations are known to those skilled in the art.

When the production of cutinase activity did no longer increase, cells were removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid and cutinase was recovered as described in Example 1.

EXAMPLE 4

Expression of Fusarium solani pisi cutinase in Aspergilli.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori an expression vector was constructed in which the synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was placed under the control of the A. niger var. awamori strong, inducible exlA promoter (Maat et al.,1992, de Graaff et al., 1992).

The pre-pro-cutinase expression plasmid (pUR7280) was constructed starting from plasmid pAW14B, which was deposited in an E. coli strain JM109 with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, under N° CBS 237.90 on 31st May 1990, and contains a ca. 5.3 kb SalI fragment on which the 0.7 kb endoxylanase II (exlA) gene is located, together with 2.5 kb of 5'-flanking sequences and 2.0 kb of 3'-flanking sequences (Fig.8). In pAW14B the exlA coding region was replaced by the pre-pro-cutinase coding region. A BspHI site (5'-TCATGA-3') comprising the first

codon (ATG) of the exlA gene and an AflII site (5'-CTTAAG-3'), comprising the stopcodon (TAA) of the exlA gene facilitated the construction of pUR7280.

The construction was carried out as follows: pAW14B (7.9 kb) was cut partially with BspHI and the linearized plasmid (7.9 kb) was isolated from an agarose gel. Subsequently the isolated 7.9 kb fragment was cut with BsmI, which cuts a few nucleotides downstream of the BspHI site of interest, to remove plasmids linearized at other BspHI sites. The fragments were separated on an agarose gel and the 7.9 kb BspHI-BsmI fragment was isolated. This was partially cut with AflII and the resulting 7.2 kb BspHI-AflII fragment was isolated.

The 0.7 kb BspHI-AflII fragment of pUR7210 comprising the entire open reading frame coding for Fusarium solani pisi pre-pro-cutinase was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7280. The constructed vector (pUR7280) can subsequently transferred to moulds (for example Aspergillus niger, Aspergillus niger var. awamori, etc) by means of conventional co-transformation techniques and the pre-pro-cutinase gene can then be expressed via induction of the endoxylanaseII promoter. The constructed rDNA vector can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin etc.) and moulds can be transformed with the resulting rDNA vector to produce the desired protein. As an example, the amdS and pyrG selection markers were introduced in the expression vector, yielding pUR7281 (Fig. 10). For this purpose a NotI site was created by converting the EcoRI site (present 1.2 kb upstream of the ATG codon of the pre-pro-cutinase gene) into a NotI site using a synthetic oligonucleotide (5'-AATTGCGGCCGC-3'), yielding pUR7282. Suitable DNA fragment comprising the entire A. nidulans amdS gene and the A. niger var. awamori pyrG gene together with their own promoters and terminators were equiped with flanking NotI sites and introduced in the NotI site of pUR7282, yielding pUR7281 (Fig. 10).

As an alternative approach for the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori, expression vectors were constructed in which a synthetic gene encoding the mature cutinase is not preceded by its own pre-pro-sequence, but by the pre-sequence of A. niger var. awamori exlA.

To prepare the synthetic cutinase gene for such fusions, several adaptor fragments were synthetized in which the coding sequence for the exlA pre-sequence is connected to the sequence encoding the N-terminus of mature cutinase in different ways. In cassette 5 this connection is made by fusing the exlA pre-sequence to the pro-sequence of cutinase. In cassette 6 the exlA pre-sequence is fused with the N-terminal residu of mature cutinase. Cassette 7 is identical with cassette 6, but here the N-terminal residue of the encoded mature cutinase polypeptide has been changed from the original Glycine into a Serine residue in order to better fit the requirements for cleavage of the signal peptide. Cassettes 5, 6 and 7 were assembled from synthetic oligonucleotides essentially as described in Example 1 (see Fig. 7). Cassette 5 was used to displace the 0.1 kb EcoRI-SpeI fragment of pUR7210, yielding pUR7287. Cassettes 6 and 7 were used to displace the 0.1 kb EcoRI-BclI fragment of pUR7210, yielding pUR7288 and pUR7289, respectively. For each of the plasmids pUR7287, pUR7288 and pUR7289 the 0.7 kb BspHI-AflII fragment was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7290, pUR7291 and pUR7292, respectively.

The constructed rDNA vectors subsequently were transferred to moulds (Aspergillus niger, Aspergillus niger var. awamori) by means of conventional co-transformation techniques and the pre-(pro)-cutinase gene were expressed via induction of the endoxylanaseII promoter. The constructed rDNA vectors can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin) and the mould can be transformed with the resulting rDNA vector to produce the desired protein, as illustrated in this example for pUR7280 (see above).

Aspergillus strains transformed with either of the expression vectors pUR7280, pUR7281, pUR7290, pUR7291, pUR7292 (containing the Fusarium solani pisi mature cutinase encoding region with or without the corresponding pro-sequence and either the cutinase signal sequence or the exlA signal sequence under the control of A. niger var. awamori exlA promoter and terminator) were grown under the following conditions: multiple 1 litre shake flasks with 400 ml synthetic media (pH 6.5) were inoculated with spores (final concentration: 10E6/ml). The medium had the following composition (AW Medium):

sucrose	10	g/l
$NaNO_3$	6.0	g/l
KCl	0.52	g/l
KH_2PO_4	1.52	g/l
$MgSO_4 \cdot 7H_2O$	0.49	g/l
Yeast extract	1.0	g/l
$ZnSO_4 \cdot 7H_2O$	22	mg/l
H_3BO_3	11	mg/l
$MnCl_2 \cdot 4H_2O$	5	mg/l
$FeSO_4 \cdot 7H_2O$	5	mg/l
$CaCl_2 \cdot 6H_2O$	1.7	mg/l
$CuSO_4 \cdot 5H_2O$	1.6	mg/l
$NaH_2MoO_4 \cdot 2H_2O$	1.5	mg/l
Na_2EDTA	50	mg/l

Incubation took place at 30°C, 200 rpm for 24 hours in a Mk X incubator shaker. After growth cells were collected by filtration (0.45 μm filter), washed twice with AW Medium without sucrose and yeast extract (salt solution), resuspended in 50 ml salt solution and transferred to 300 ml shake flasks containing 50 ml salt solution to which xylose has been added to a final concentration of 10 g/l (induction medium). Incubation under the same conditions as described above was continued overnight. The resulting cultures were filtered over miracloth to remove biomass and cutinase was recovered essentially as described in Example 2.

EXAMPLE 5

Identification and isolation of genes related to the Fusarium solani pisi cutinase gene.

Genes encoding cutinases with a varying degree of homology with Fusarium solani pisi cutinase were isolated from different fungi. Fungal cultures were grown in 500 ml shakeflasks containing 200 ml of the medium described by Hankin and Kolattukudy (1968) supplemented with 0.25% glucose and incubated for 4 days at 28°C in a Mk X incubator shaker (100 rpm). At this time the glucose had been consumed and cutinase production was induced by the addition of cutin hydrolysate essentially as described by Ettinger et al. (1987). At regular intervals samples were withdrawn from the culture and analyzed for the presence of lipolytic activity according to standard techniques (see example 4). Normally, about two days after induction lipolytic activity could be demonstrated and at that time the cells were harvested by filtration using standard techniques. The mycelia were washed, frozen in liquid nitrogen and lyophilized according to standard techniques. Total cellular RNA preparations were isolated using the guanidinium thiocyanate method and purified by cesium chloride density gradient centrifugation, essentially as described by Sambrook et al. (1989). PolyA(+) mRNA fractions were isolated using a polyATtract mRNA isolation kit (Promga). The polyA(+) mRNA fractions were used in a Northern hybridization analysis using a cDNA fragment from the Fusarium solani pisi cutinase gene as a probe according to standard techniques, to verify the expression of cutinase-related genes. Preparations of mRNA comprising material capable of hybridizing with the probe were used for the synthesis of cDNA using a ZAP cDNA synthesis kit (Stratagene, La Jolla) according to the instructions of the supplier, yielding cDNA fragments with an XhoI cohesive end flanking the poly-A region and an EcoRI adaptor at the other end. The obtained cDNA fragments were used for the construction of expression libraries by directional cloning in the sense orientation in lambda ZAPII vectors (Stratagene, La Jolla), allowing expression of ß-galactosidase fusion

proteins (Huse et al.,1988). These libraries were screened using antiserum raised against Fusarium solani pisi cutinase.

Alternatively, the synthesized cDNA fractions were subjected to PCR-screening using cutinase specific primers (see table 2). These primers were derived from comparison of the amino acid sequence of several fungal Cutinase genes (Ettinger et al., 1987). The conditions for the PCR reaction were optimized for each set of primers, using cDNA from Fusarium solani pisi cutinase as a control. For those preparations of cDNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under identical conditions, the PCR fragment was purified by gel electroforesis and isolated from the gel.

As an alternative approach, the PCR screening technique using cutinase specific primers was also applied directly to genomic DNA of some fungal strains, using genomic DNA of Fusarium solani pisi as a positive control. For those preparations of fungal genomic DNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under identical conditions, the PCR fragment was purified by gel electrophoresis and isolated from the gel.

For strains which scored positive in either the expression library approach or the PCR screening approach (either with cDNA or genomic DNA) as well as a number of other strains, high molecular weight genomic DNA was isolated. Strains were grown essentially as described by Ettinger et al. (1987), and genomic DNA was isolated as described by de Graaff et al. (1988). Genomic DNA was digested with various restriction enzymes and analyzed by Southern hybridization using either the analogous cDNA insert (expression library approach) or the PCR fragment (PCR screening approach) or the Fusarium solani pisi cutinase gene (other strains) as a probe, and a physical map of the cutinase genes was constructed. An appropriate digest of

genomic DNA was size-fractionated by gel electrophoresis and fragments of the appropriate size were isolated from the gel and subcloned in pUC19. These genomic libraries were screened with the corresponding cDNA insert (expression library approach) or the PCR fragment (PCR screening approach), yielding clones comprising the genomic copy of the cutinase genes. These genes were sequenced in both directions. Introns were identified by sequencing the corresponding cDNA or by comparison with other cutinase sequences (Ettinger et al., 1987). The N-terminal end of the mature cutinase polypeptide was also deduced from such a comparison. Using standard PCR techniques, the introns were removed, a HindIII site was engineered immediately downstream of the open reading frames and the coding sequence for the pre-sequence of the Saccharomyces cerevisiae invertase gene (preceded by a SacI site, compare cassette 8, Fig. 3) was fused to the sequences encoding the N-terminus of the mature cutinases. The obtained SacI-HindIII fragments comprising the cutinase genes operably linked to the sequence encoding the S. cerevisiae invertase pre-sequence were ligated with the 7.3 kb SacI-HindIII fragment of pUR7241 (see Fig. 4) and transformed to S. cerevisiae strain SU51. The fungal cutinases were expressed and recovered from the culture broth essentially as described in Example 4.

EXAMPLE 6

Inactivation experiments with Di-isopropyl Fluoro Phosphate (DFP).

In this experiment lipolytic enzymes of various origins were subjected to inactivation by various concentrations of DFP for a fixed period of time. The percentage rest activity after the inactivation period was measured in a pH-stat experiment.

The experimental conditions were:

The lipolytic enzyme to be tested was used at a concentration of 0.5 mg protein per ml in 10 mM Tris buffer pH 10.0 containing 20 mM $CaCl_2.2H_2O$ (Merck). 20 µl DFP-inhibitor solution was added to 0.5 ml of this enzyme solution

(sample), and 20 µl ether was added to another 0.5 ml enzyme solution (blank). The DFP-inhibitor solution was 0.5 M DFP (Di-isopropyl Fluoro Phosphate ex Fluka) in ether (Baker). Both samples were incubated for 10 minutes at room temperature. After the incubation the solution was centrifuged for 2 minutes at full speed (14,000 rpm) in an Eppendorf centrifuge. The supernatant was used. In both the sample and the blank the lipase activity was measured in a pH-stat experiment at pH 10.0, using Lipase substrate (ex Sigma, catalogue no. 800-1). The residual activity (RA) was then calculated as follows:

RA = lipase activity sample / lipase activity blank * 100 %

The percentages residual activity are shown below:

Lipolytic enzyme	residual activity (%)
Lipolase (TM, ex Novo Nordisk)	86
Fusarium solani pisi cutinase (Example 2)	5
Candida cylindracea lipase (ex Sigma)	83
Chromobacterium viscosum lipase (ex Sigma)	65
Porcine pancreas lipase (ex Sigma)	60
Genencor lipase (variant of Pseudomonas putida ATCC 53552, "Lumafast")	65
AKG lipase 30B (ex Amano)	52
SDL 195 lipase (ex Showa Denko)	20
Ps. pseudoalcaligines CBS 473.85 lipase	60

EXAMPLE 7

The lipolytic activity of the enzymes tested in the previous Example was measured using the Br-olive oil method described above. The washing temperature was 30°C and the water hardness was 27°FH. The percentages Br-olive oil removed in the washing experiment were as follows:

Lipolytic enzyme	Soil Removal (%)
Lipolase (TM, ex Novo Nordisk)	0
Fusarium solani pisi cutinase (Example 2)	19
Candida cylindracea lipase (ex Sigma)	0
Chromobacterium viscosum lipase (ex Sigma)	1.6

Porcine pancreas lipase (ex Sigma)	10
Genencor lipase (variant of Pseudomonas putida ATCC 53552)	11
AKG lipase 30B (ex Amano)	10
SDL 195 lipase (ex Showa Denko)	20
Ps. pseudoalcaligines CBS 473.85 lipase	15

The data obtained in Example 6 (residual activity after inactivation with DFP) and the data obtained in this Example were correlated to each other by means of lineair regression analysis. In Figure 11 a graphical representation of this correlation is shown. It can be seen in this Figure that there is a good correlation between the percentage residual activity and the wash performance of a given lipolytic enzyme. We therefore conclude that the percentage residual activity after incubation with DFP according to Example 6 can be used as a simple predictive test for in-the-wash performance of lipolytic enzymes of any source.

EXAMPLE 8

Comparison of the lipolytic activity of Fusarium solani pisi cutinase and Lipolase (TM).

The lipolytic activity of cutinase from Fusarium solani pisi, which was isolated according to Example 2, was compared with the lipolytic activity of Lipolase (TM), a Humicola lanuginosa lipase, commercially available from Novo Nordisk A/S.

Test cloths made of woven cotton and knitted cotton were soiled with pure olive oil. Each tests cloth was then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The bottles were agitated in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The wash liquor consisted of 2 grams per litre (at 6° FH) of a washing powder having the following composition (in % by weight):

Nonionic surfactant C_{12}-C_{15} alcohol 10.5-13EO	9.5
Sodium sulphate	38.6
Sodium carbonate	40.4

Sodium silicate ($Na_2O:Si_2O$ = 2.4)	7.3
Water	4.2

Residual fat was determined immediately after the first wash, after drying the test cloths in a tumble dryer at ambient temperature. The test cloths were extracted in a Soxhlet apparatus with petroleum ether. Subsequently, the amount of fatty material was determined by weighing and was expressed as the percentage fat on the cloth. The results are given in Figure 12.

It can be seen from this Figure that Lipolase (TM) gives no improvement over the control in the removal of olive oil; in the experiment shown the contribution was sligthly negative but this is not believed to be significant. Cutinase, on the other hand, shows a clear in-the-wash effect.

EXAMPLE 9

Single-wash effect of Fusarium solani pisi cutinase and Lipolase (TM) on different types of soiling.

Test cloths made of cotton were soiled with peanut oil, oleyl oleate and a 50/50 mixture of these two fats. The washing powder and the washing conditions were the same as in Example 8. The cleaning effect after a single wash was assessed by measuring the reflectance at 460 nm. The results are given in Figure 13.

It can be seen from this Figure that the cleaning effect of a detergent composition containing cutinase on several types of oily soil is consistently better than that of a composition containing Lipolase (TM). The effect is most pronounced on pure peanut oil.

EXAMPLE 10

The effects of Fusarium solani pisi cutinase and Lipolase (TM) at a number of enzyme levels, measured after each wash cycle.

Example 9 was essentially repeated whereby the test cloths were soiled with LS-3 soil. This is an emulsion of 75 g peanut oil, 40 g emulsifier, 125 g AS-8 and 125 g milk

powder. After the first wash, the test cloths were resoiled and washed again. This was repeated four times in total. The effect of cutinase and Lipolase (TM) was measured at an enzyme level of 1, 3, 5 and 10 LU per ml wash liquor, after each wash cycle. The results are given in Figure 14 A-D.

Several conclusions can be drawn from these experiments. It is clear that cutinase shows a significant single-wash effect at all enzyme levels, whereas Lipolase (TM) shows only a minor single-wash effect at the highest level of 10 LU/ml. The advantage of cutinase over Lipolase (TM) after the first wash is sustained over all four wash cycles.

EXAMPLE 11

The effects of Fusarium solani pisi cutinase, lipase ex Pseudomonas gladioli and Lipolase (TM).

Test cloths of 67% polyester and 33% cotton were desized using the detergent product shown below at 5 g/l, and were thoroughly rinsed. Pieces of 7.5 cm by 7.5 cm were cut and overlocked. Ten of such unsoiled cloths (total mass approximately 6.5 g) were washed in 75 ml wash liquor containing the detergent product given below at a dosage of 5 g per litre, at 6° FH. Lipase ex Pseudomonas gladioli, Lipolase or Fusarium solani pisi cutinase were preadded to the wash liquor at 1 LU/ml. The lipase ex Pseudomonas gladioli was obtained as described in EP-A-205 208 and EP-A-206 390 (both Unilever). The wash liquor and the cloths were held in small bottles which were agitated in a Lavamat AEG washing machine, at 30°C for 30 minutes. The washing powder had the following composition (in % by weight):

Coco-primary alkyl sulphate	5.2
Nonionic surfactant C_{13}-C_{15} alcohol 7EO	5.2
Nonionic surfactant C_{13}-C_{15} alcohol 3EO	6.6
Zeolite 4A	32.00
Sodium carbonate	11.52
Sodium silicate	0.45
Hardened tallow soap	2.00

The initial pH was in all cases about 10. At the end of the wash the cloths were squeezed dry and rinsed in 300 ml of tap water for about half a minute. This was repeated three times. The cloths were then squeezed dry once more and line dried under ambient conditions.

Half of the dried cloths were soiled with olive oil and aged for three days. The mass of oily soil which was initially approximately 5% by weight of the cloth was determined by weighing. The results are given in Figure 15.

It is clear that Lipolase (TM) does not contribute to oily soil removal after a single wash. The Lipolase (TM) effect comes only through after repeated washing. The P. gladioli lipase gives a small but significant wash effect after the first wash and a clear benefit after subsequent wash cycles. Cutinase gives already contributes to oily soil removal from the first wash cycle, and this benefit is maintained through the subsequent cycles.

EXAMPLE 12

The effects of Fusarium solani pisi cutinase, lipase ex Pseudomonas gladioli and Lipolase (TM).

Example 11 was repeated using the following detergent product at a concentration of 5 g/l:

Coco-primary alkyl sulphate	1.7
Nonionic surfactant C_{12}-C_{15} alcohol 7EO	6.8
Nonionic surfactant C_{12}-C_{15} alcohol 3EO	8.5
Zeolite MAP [1])	32.00
Sodium carbonate	11.52
Sodium silicate	0.45
Hardened tallow soap	2.00

[1]) Zeolite MAP stands for "maximum aluminium zeolite P"; it is a type of zeolite which is described in detail in EP-A-384 070 (Unilever).

The results are given in Figure 16. It follows that both lipases do not contribute to oily soil removal, neither after the first wash, nor after subsequent cycles. Cutinase,

however, already gives a large in-the-wash effect after the first wash cycle.

EXAMPLE 13

The effects of Fusarium solani pisi cutinase, lipase ex Pseudomonas gladioli and Lipolase (TM).

Example 12 was repeated, but with resoiling between the wash cycles. The results are given in Figure 17. A small benefit is observed Lipolase (TM) and P. gladioli lipase after the second wash cycle. Cutinase already reduces the amount of oily soil to a minimum in a single wash. Subsequent wash cycles result in a further reduction of oily soil, even after resoiling.

CLAIMS

1. An enzymatic detergent composition which comprises:

(a) 0.1 - 50 % by weight of an surfactant system comprising

(a1) 0 - 95 % by weight of one or more anionic surfactants and

(a2) 5 - 100 % by weight of one or more nonionic surfactants; and

(b) 10 - 20,000 LU per gram of the detergent composition of an enzyme which is capable of exhibiting a substantial lipolytic activity during the main cycle of a wash process.

2. A detergent composition according to Claim 1, in which the enzyme is capable of removing at least 5% more, preferably at least 10% more oily soil, on the basis of the initial amount of oily soil, than the same detergent composition without the enzyme, in the assay as herein described.

3. A detergent composition according to Claim 1, in which the ratio of enzymatic oily soil removal by the enzyme to the enzymatic oily soil removal by Lipolase (TM) in the assay as herein described is at least 3, preferably at least 5.

4. A detergent composition according to any one of the preceding claims, wherein the enzyme exhibiting lipolytic activity is a lipolytic enzyme which has a residual activity of less than 50%, preferably less than 40%, and more preferably less than 25%, after a 10 minute incubation with Di-isopropyl Fluoro Phosphate (DFP) at pH 10 at room temperature, as herein described.

5. A detergent composition according to any one of the preceding claims, in which the enzyme is an esterase or lipase.

6. A detergent composition according to any one of the preceding claims, in which the enzyme is a eukaryotic cutinase.

7. A detergent composition according to Claim 6, in which the enzyme is a fungal cutinase.

8. A detergent composition according to Claim 7, in which the enzyme is a fungal cutinase having stem-specificity.

9. A detergent composition according to Claim 8, in which the enzyme is a cutinase selected from the group consisting of Fusarium solani pisi, Fusarium roseum culmorum, Rhizoctonia solani and Alternaria brassicicola (PNBase I).

10. A detergent composition according to Claim 7, in which the enzyme is a cutinase obtainable from a Fusarium organism.

11. A detergent composition according to Claim 7, in which the enzyme is a cutinase derived from Fusarium solani pisi.

12. A detergent composition according to Claim 7, in which the enzyme is immunologically cross-reactive with the antibody raised against the cutinase derived from Fusarium solani pisi.

13. A detergent composition according to any one of the preceding claims, free of anionic surfactants.

14. A detergent composition according to Claims 1-12, in which the anionic surfactant is a primary alcohol sulphate.

15. A detergent composition according to Claim 14, in which the anionic surfactant is a C12-C15 primary alcohol sulphate.

16. Process for cleaning soiled fabrics which comprises the steps of (a) washing the soiled fabrics with an aqueous solution of the detergent composition according to any one of the preceding claims, and (b) drying the fabrics by means of warm air.

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Fig.1A.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE I

CODE	Length	5' <--- sequence ---> 3'
CUTI1A IG	(44)	aat tcg agc tca tcA TGA AAT TCT TCG CGT TAA CCA CAC TTC TC
CUTI1B IG	(39)	GCC GCC ACG GCT TCG GCT CTG CCT ACT AGT AAC CCT GCT
CUTI1C IG	(42)	CAG GAG CTT GAG GCG CGC CAG CTT GGT AGA ACA ACT CGC GAC
CUTI1D IG	(39)	GAT CTG ATC AAC GGC AAT AGC GCT TCC TGC GCC GAT GTC
CUTI1E IG	(33)	ATC TTC ATT TAT GCT CGA GGT TCA ACA GAG ACG
CUTI1F IG	(28)	GGC AAC TTG GGA ACT CTC GGG CCC AGC A
CUTI1G IG	(31)	GGT TAA CGC GAA GAA TTT CAT gat gag ctc g
CUTI1H IG	(39)	ACT AGT AGG CAG AGC CGA AGC CGT GGC GGC GAG AAG TGT
CUTI1I IG	(42)	TGT TCT ACC AAG CTG GCG CGC CTC AAG CTC CTG AGC AGG GTT
CUTI1J IG	(39)	GCA GGA AGC GCT ATT GCC GTT GAT CAG ATC GTC GCG AGT
CUTI1K IG	(33)	TGA ACC TCG AGC ATA AAT GAA GAT GAC ATC GGC
CUTI1L IG	(41)	AGC TTG CTG GGC CCG AGA GTT CCC AAG TTG CCC GTC TCT GT

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                  gc tcgagtagTA CTTTAAGAAG CGCAATTGGT GTGAAGAGCG

        70          80          90          100         110         120
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GCGGTGCCGA AGCCGAGACG GATGATCATT GGGACGAGTC CTCGAACTCC GCGCGGTCGA

       130         140         150         160         170         180
TGGTAGAACA ACTCGCGACG ATCTGATCAA CGGCAATAGC GCTTCCTGCG CCGATGTCAT
ACCATCTTGT TGAGCGCTGC TAGACTAGTT GCCGTTATCG CGAAGGACGC GGCTACAGTA

       190         200         210         220         230         240
CTTCATTTAT GCTCGAGGTT CAACAGAGAC GGGCAACTTG GGAACTCTCG GGCCCAGCA
GAAGTAAATA CGAGCTCCAA GTTGTCTCTG CCCGTTGAAC CCTTGAGAGC CCGGGTCGTT

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GGA
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Fig.1B.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 2

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CUTI2B MH	(36)	TTC GGC AAG GAC GGT GTC TGG ATT CAG GGC GTT GGC
CUTI2C MH	(36)	GGT GCC TAC CGA GCC ACC CTA GGA GAC AAT GCT CTC
CUTI2D MH	(39)	CCG CGG GGA ACC TCT AGC GCC GCA ATC AGG GAG ATG CTA
CUTI2E MH	(45)	GGC CTC TTC CAG CAG GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG
CUTI2F MH	(46)	ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA CTT GCA GCC GCT AGC A
CUTI2G MH	(45)	CTT GCC GAA GGC GGA CTC AAG GTT GGA GGC AAT GCT GGG CCC GAG
CUTI2H MH	(36)	GTA GGC ACC GCC AAC GCC CTG AAT CCA GAC ACC GTC
CUTI2I MH	(36)	TCC CCG CGG GAG AGC ATT GTC TCC TAG GGT GGC TCG
CUTI2J MH	(39)	GAA GAG GCC TAG CAT CTC CCT GAT TGC GGC GCT AGA GGT
CUTI2K MH	(45)	ACC GGC GAT CAA AGT CGC GTC AGG GCA CTT GGT GTT GGC CTG CTG
CUTI2L MH	(41)	AGC TTG CTA GCG GCT GCA AGT GCA GCA CCC TGG CTG TAG CC

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      70         80         90        100        110        120
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GACCTAAGTC CCGCAACCGC CACGGATGGC TCGGTGGGAT CCTCTGTTAC GAGAGGCGC

     130        140        150        160        170        180
GGGAACCTCT AGCGCCGCAA TCAGGGAGAT GCTAGGCCTC TTCCAGCAGG CCAACACCAA
CCCTTGGAGA TCGCGGCGTT AGTCCCTCTA CGATCCGGAG AAGGTCGTCC GGTTGTGGTT

     190        200        210        220        230        240
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CACGGGACTG CGCTGAAACT AGCGGCCACC GATGTCGGTC CCACGACGTG AACGTCGGCG

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ATCGTTCGA
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Fig.1C.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 3

CODE	Length	5' <--- sequence ---> 3'
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CUTI3C1 MH	(42)	AAT CGC GGC CGA ATC CCC AAC TAC CCT GCC GAC AGG ACC AAG
CUTI3D MH	(42)	GTC TTC TGC AAT ACA GGA GAT CTC GTT TGT ACT GGT AGC TTG
CUTI3E MH	(39)	ATC GTT GCT GCA CCT CAC TTG GCA TAT GGT CCT GAT GCC
CUTI3F MH	(33)	CGG GGA CCT GCC CCT GAG TTC CTC ATC GAG AAG
CUTI3G1 MH	(32)	GTT CGG GCT GTC CGT GGT TCT GCT TGA gct ta
CUTI3H MH	(30)	GGC CGA GTC GAG GTC CTC GAT GCT AGC GGG
CUTI3I MH	(45)	CTT GGT GTA GCC GAA CAG AAC GGT ACC GGC GAT CTT GTC ACG AAT
CUTI3J1 MH	(42)	GTC GGC AGG GTA GTT GGG GAT TCG GCC GCG ATT CTG TAG GTT
CUTI3K MH	(42)	AGT ACA AAC GAG ATC TCC TGT ATT GCA GAA GAC CTT GGT CCT
CUTI3L MH	(39)	ACC ATA TGC CAA GTG AGG TGC AGC AAC GAT CAA GCT ACC
CUTI3M MH	(33)	GAG GAA CTC AGG GGC AGG TCC CCG GGC ATC AGG
CUTI3N1 MH	(45)	agc tta agc TCA AGC AGA ACC ACG GAC AGC CCG AAC CTT CTC GAT

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        70          80          90          100         110         120
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        130         140         150         160         170         180
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TGTCCTGGTT CCAGAAGACG TTATGTCCTC TAGAGCAAAC ATGACCATCG AACTAGCAAC

        190         200         210         220         230         240
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        250         260         270         280
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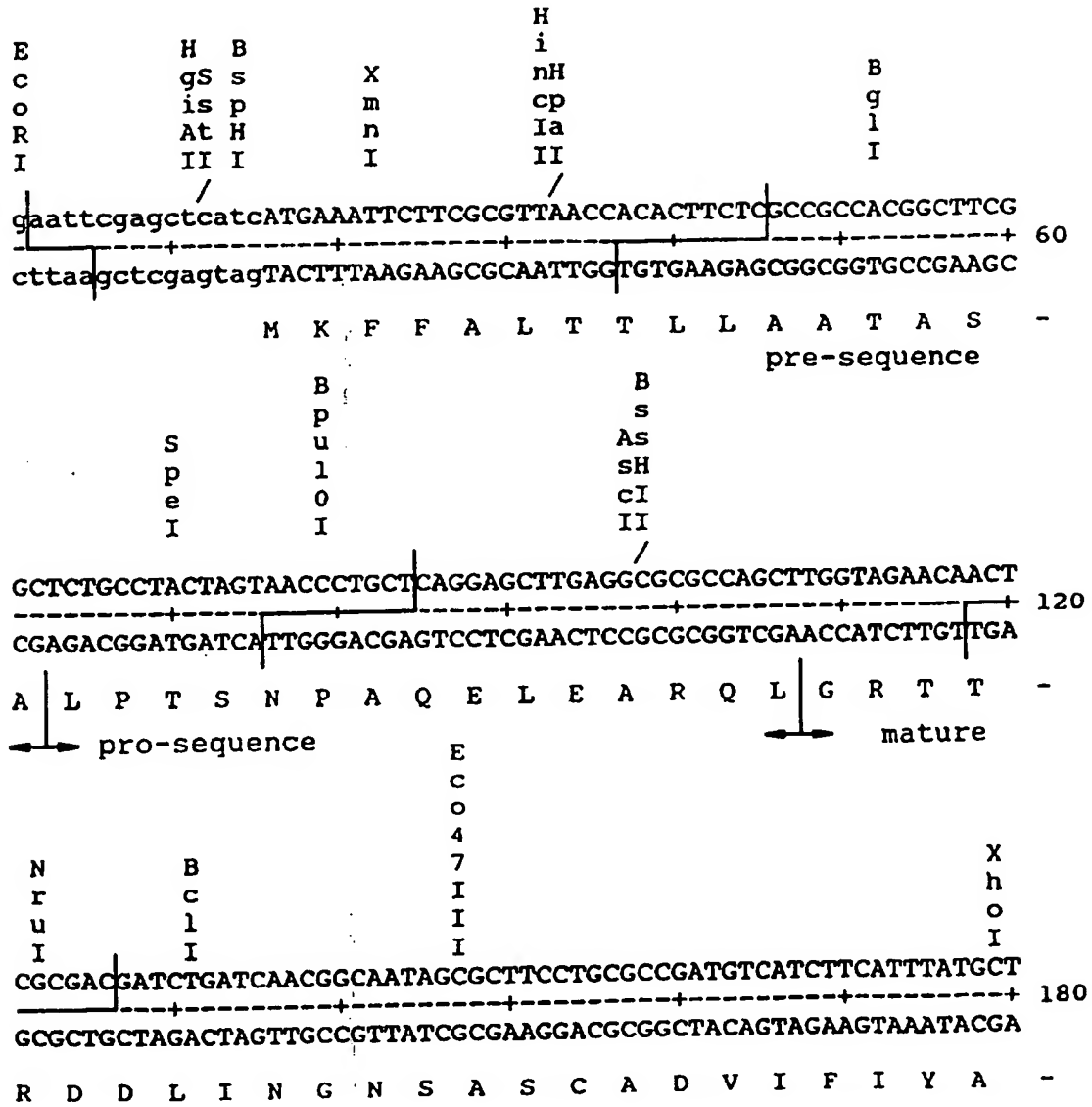
Fig.1D(1 of 4).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mature
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BclI
Eco47III
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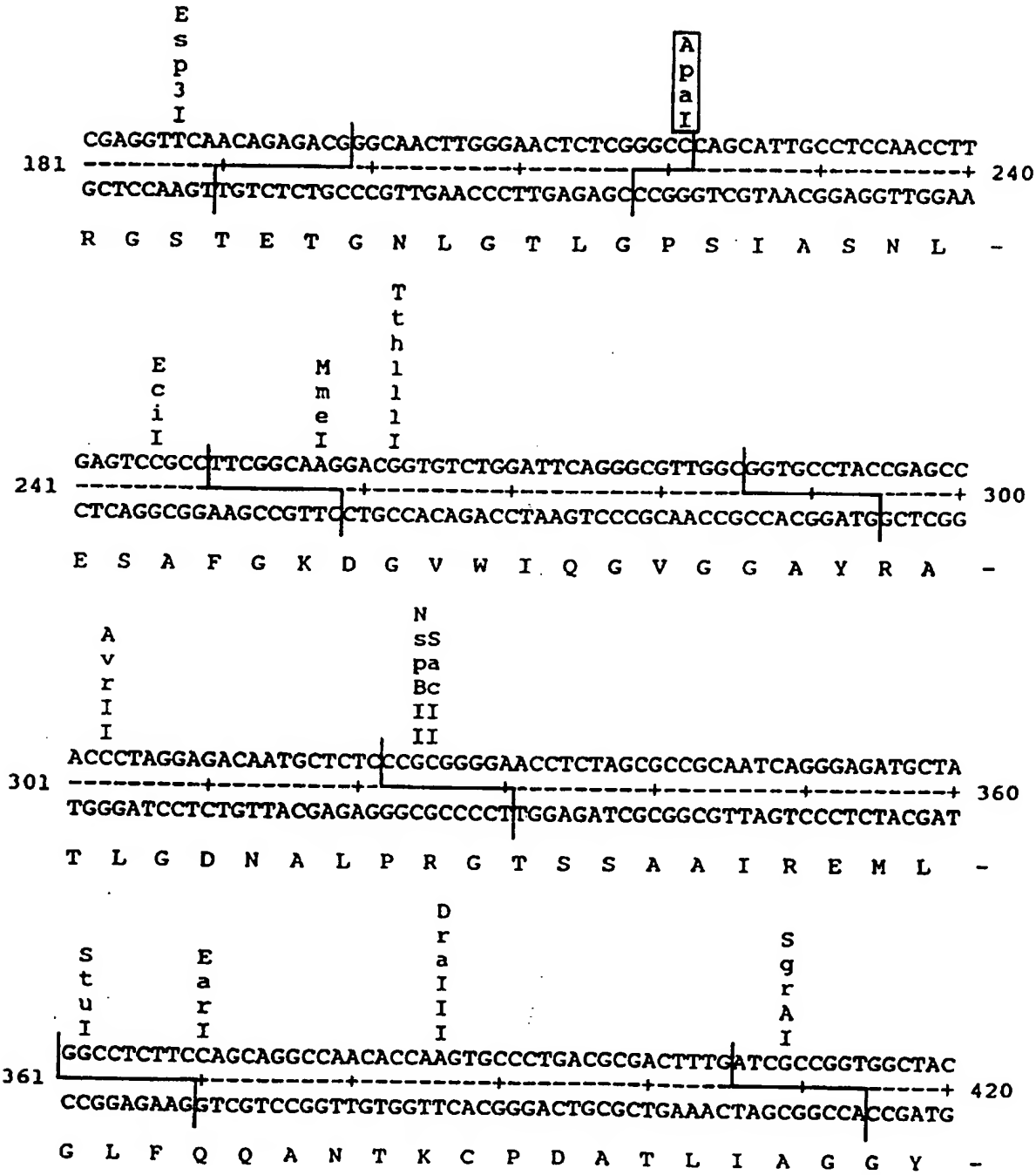
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Fig.1D(3 of 4)

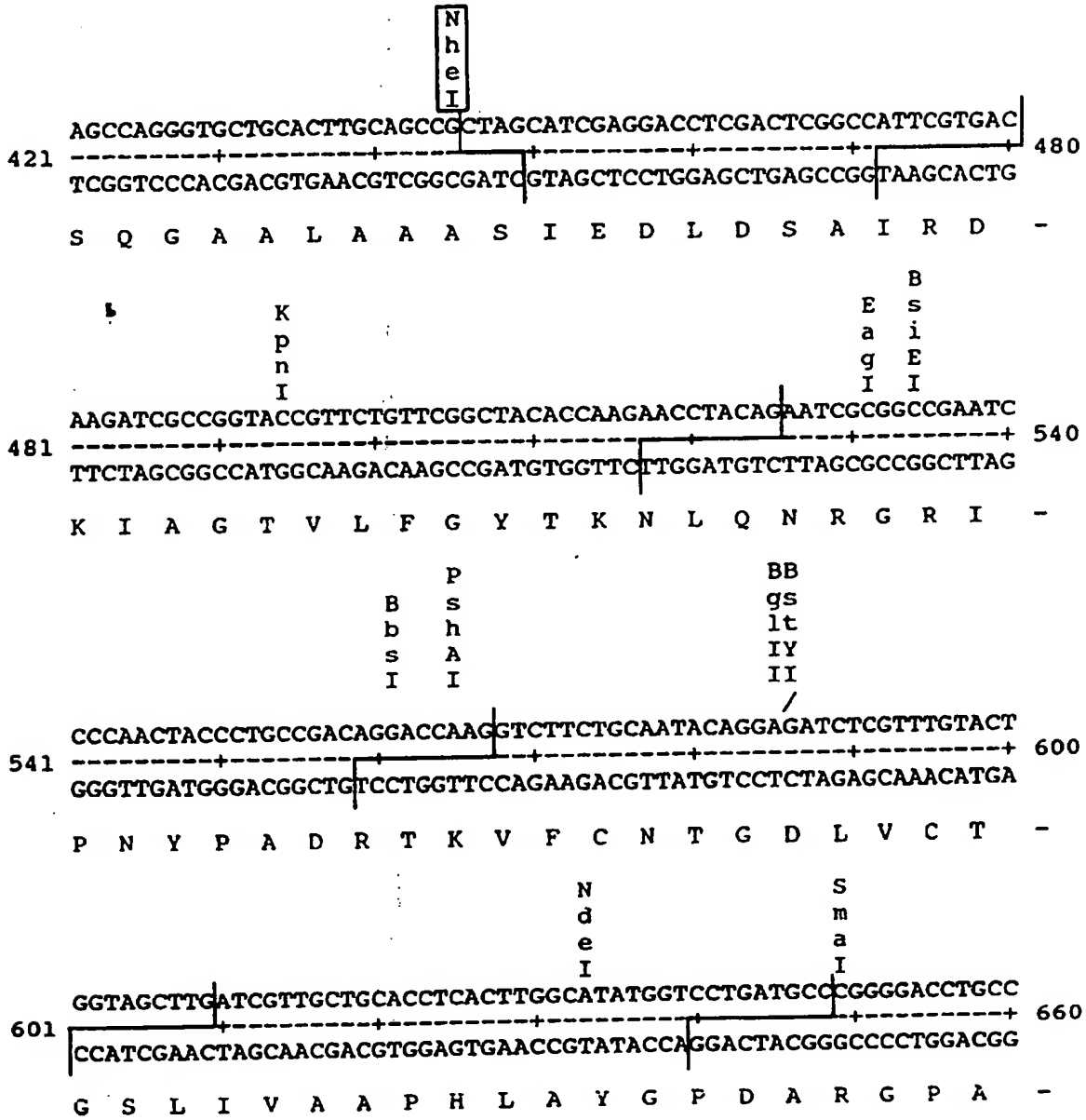
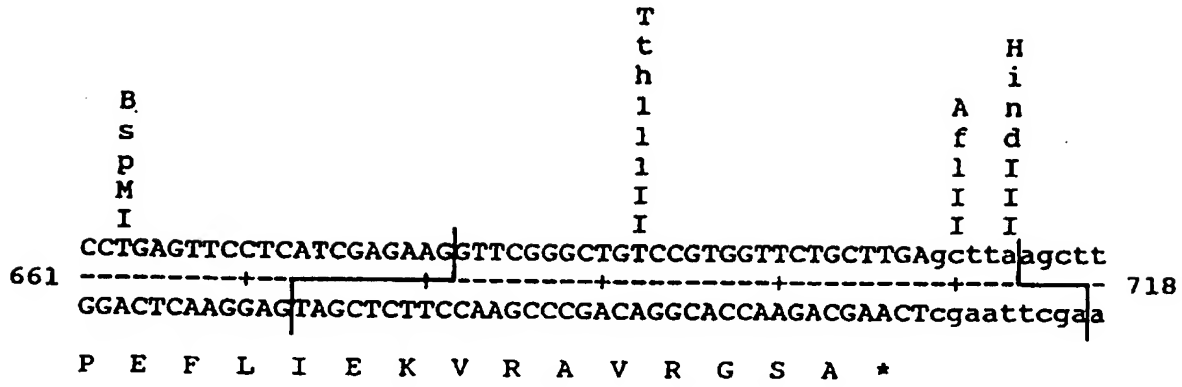


Fig.1D(4 of 4)


T
t
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H
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A
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d
p
l
I
I
M
I
I
I
I
I
I
CCTGAGTTCCTCATCGAGAAGGTTCGGGCTGTCCGTGGTTCTGCTTGAgcttaagctt
661
718
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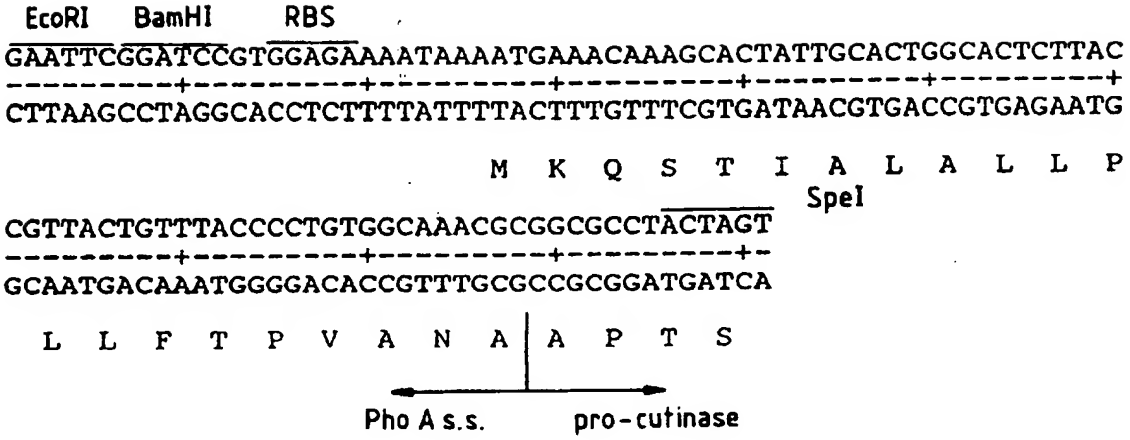
P E F L I E K V R A V R G S A *

Fig. 2.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BamHI
RBS
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CTTAAGCCTAGGCACCTCTTTTATTTTACTTTGTTTCGTGATAACGTGACCGTGAGAATG
M K Q S T I A L A L L P
SpeI
CGTTACTGTTTACCCCTGTGGCAAACGCGGCGCCTACTAGT
GCAATGACAAATGGGGACACCGTTTGCGCCGCGGATGATCA
L L F T P V A N A A P T S
Pho A s.s.
pro-cutinase

Fig. 3.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 8

CODE	Length	5' <--- sequence ---> 3'
		SacI
AC 01 cv	(38)	AAT TCT CGA GCT CAT CAC ACA AAC AAA CAA
		AAC AAA AT
AC 02 CV	(25)	GAT GCT TTT GCA AGC CTT CCT TTT C
AC 03 CV	(39)	CTT TTG GCT GGT TTT GCA GCC AAA ATA TCT
		GCG GGT AGA
		BclI
AC 04 CV	(25)	ACA ACT CGC GAC GAT CTG ATC ATC A
AC 05 CV	(41)	AGC TTG ATG ATC AGA TCG TCG CGA GTT GTT
		CTA CCC GCA GA
AC 06 CV	(17)	TAT TTT GGC TGC AAA AC
AC 07 CV	(46)	CAG CCA AAA GGA AAA GGA AGG CTT GCA AAA
		GCA TCA TTT TGT TTT G
AC 08 CV	(23)	TTT GTT TGT GTG ATG AGC TCG AG

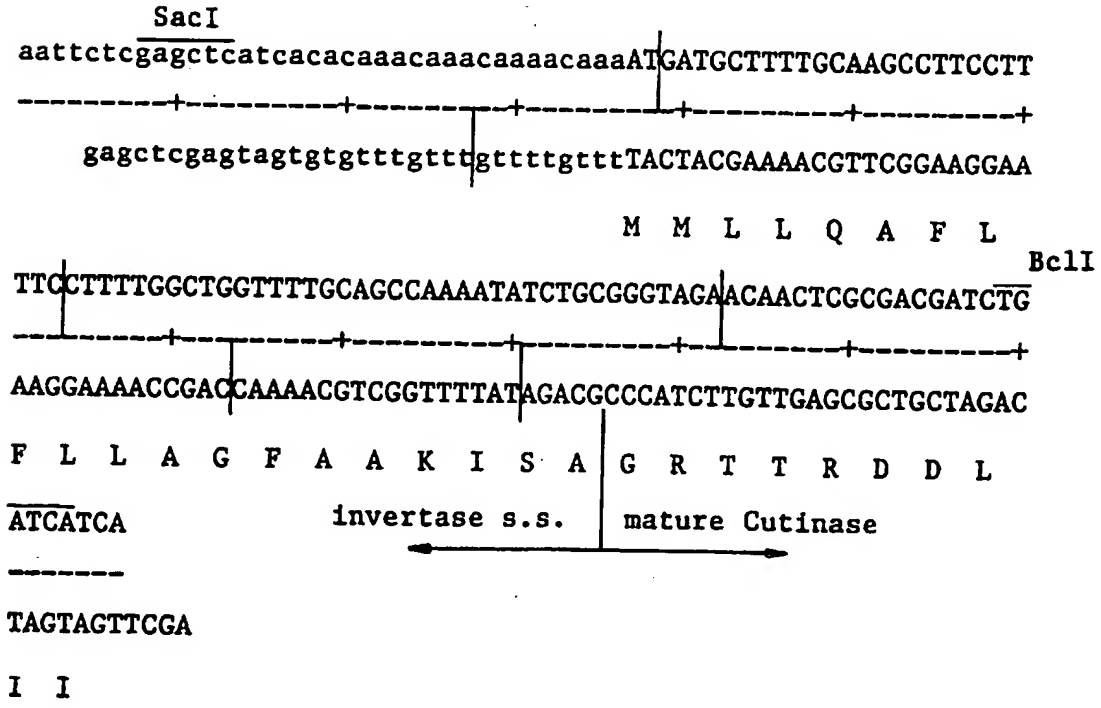
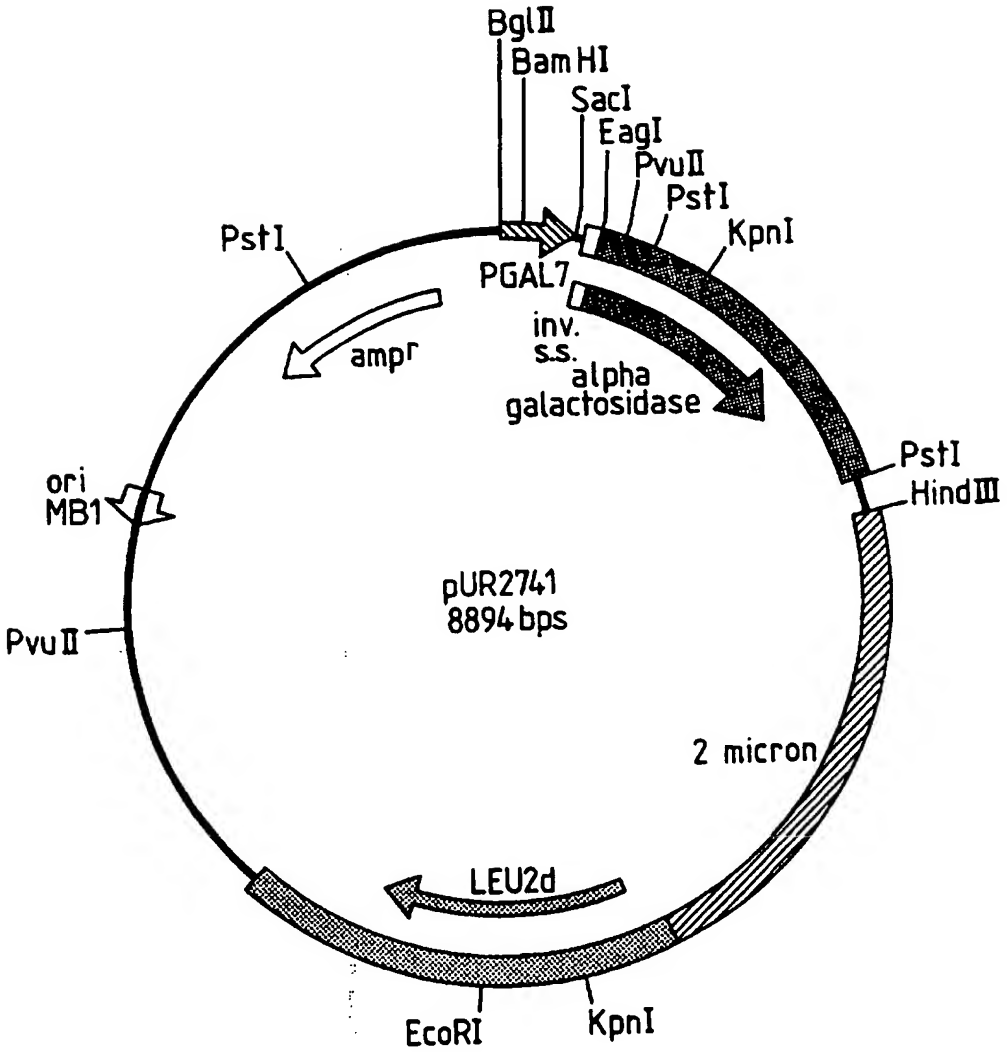


Fig.4.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BamHI
SacI
EagI
PvuII
PstI
KpnI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
ori
MB1
PstI
HindIII
pUR2741
8894 bps
PvuII
2 micron
LEU2d
EcoRI
KpnI

Fig. 5.

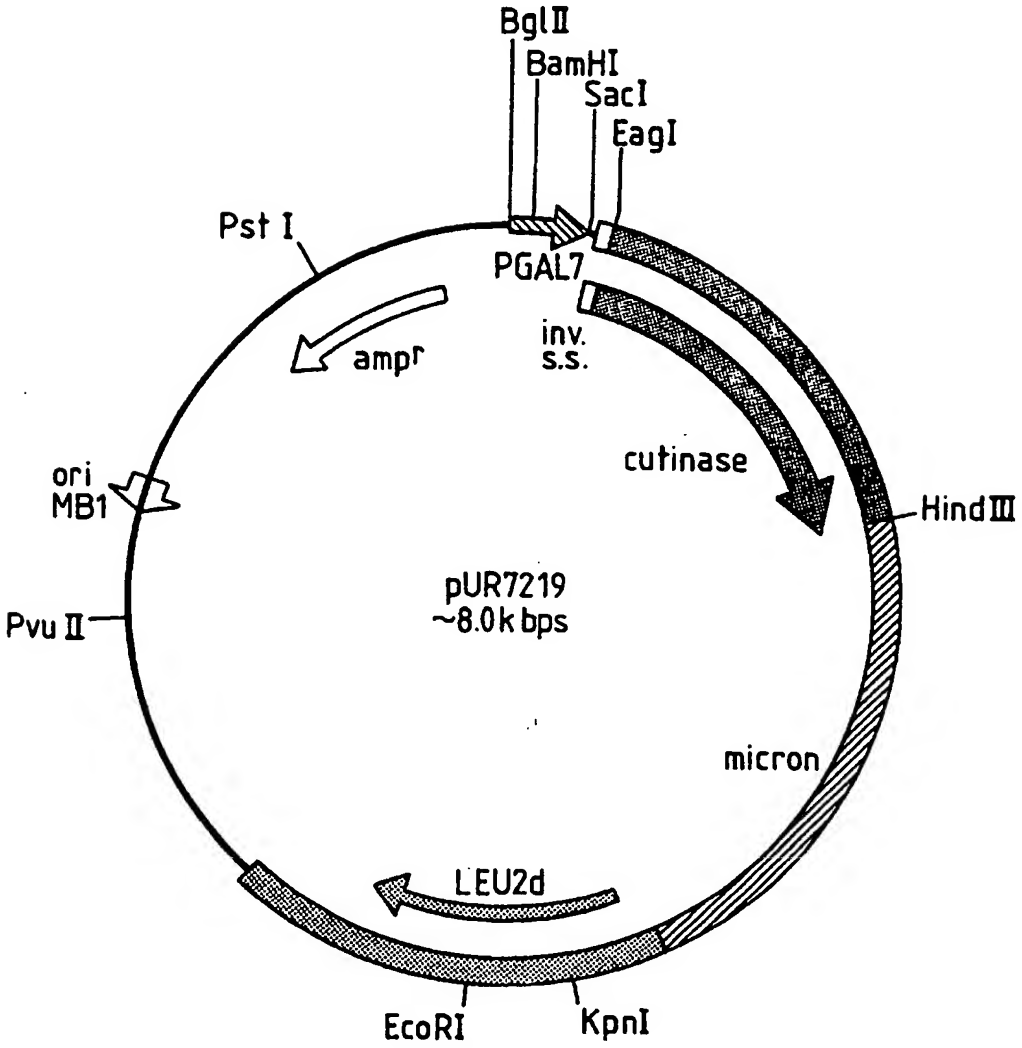

BglII
BamHI
SacI
EagI
Pst I
PGAL7
inv.
s.s.
ampr
cutinase
ori
MB1
HindIII
pUR7219
~8.0k bps
Pvu II
micron
LEU2d
EcoRI
KpnI

Fig. 6.

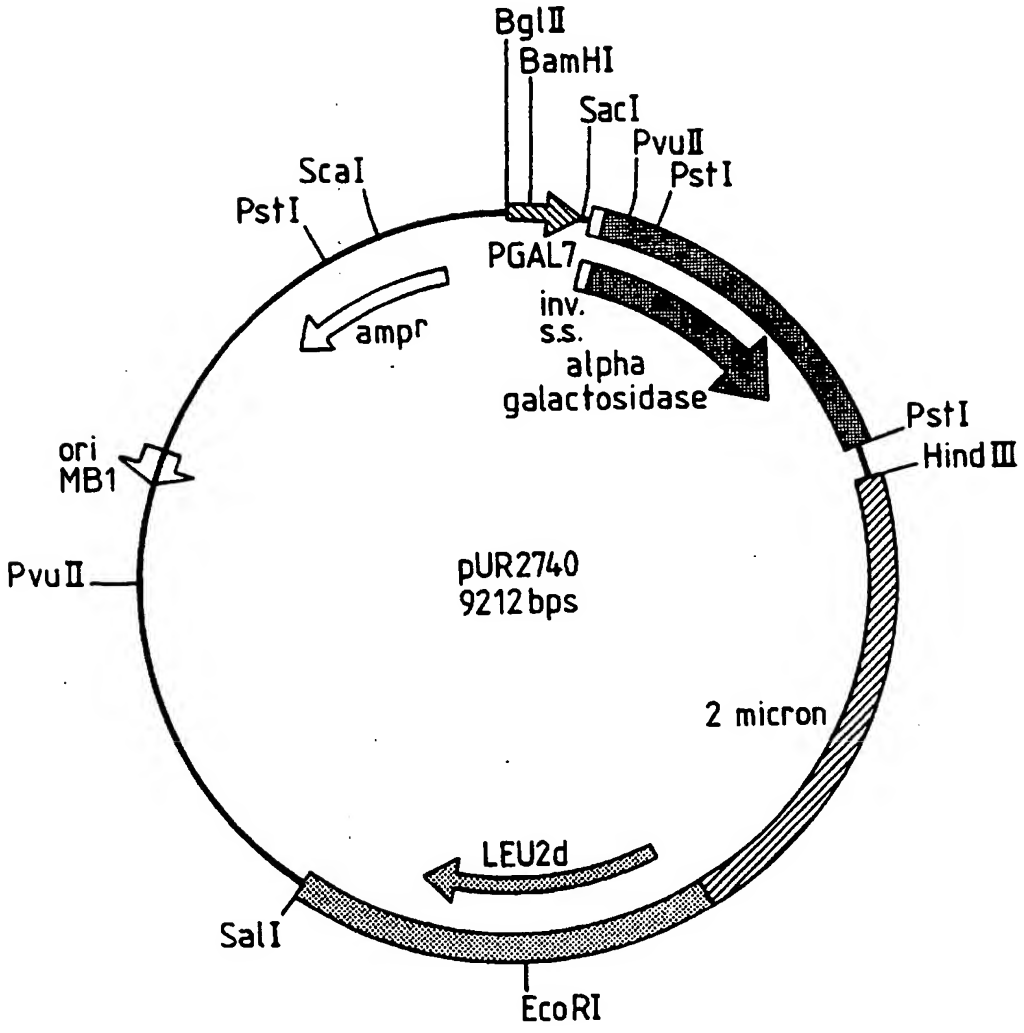
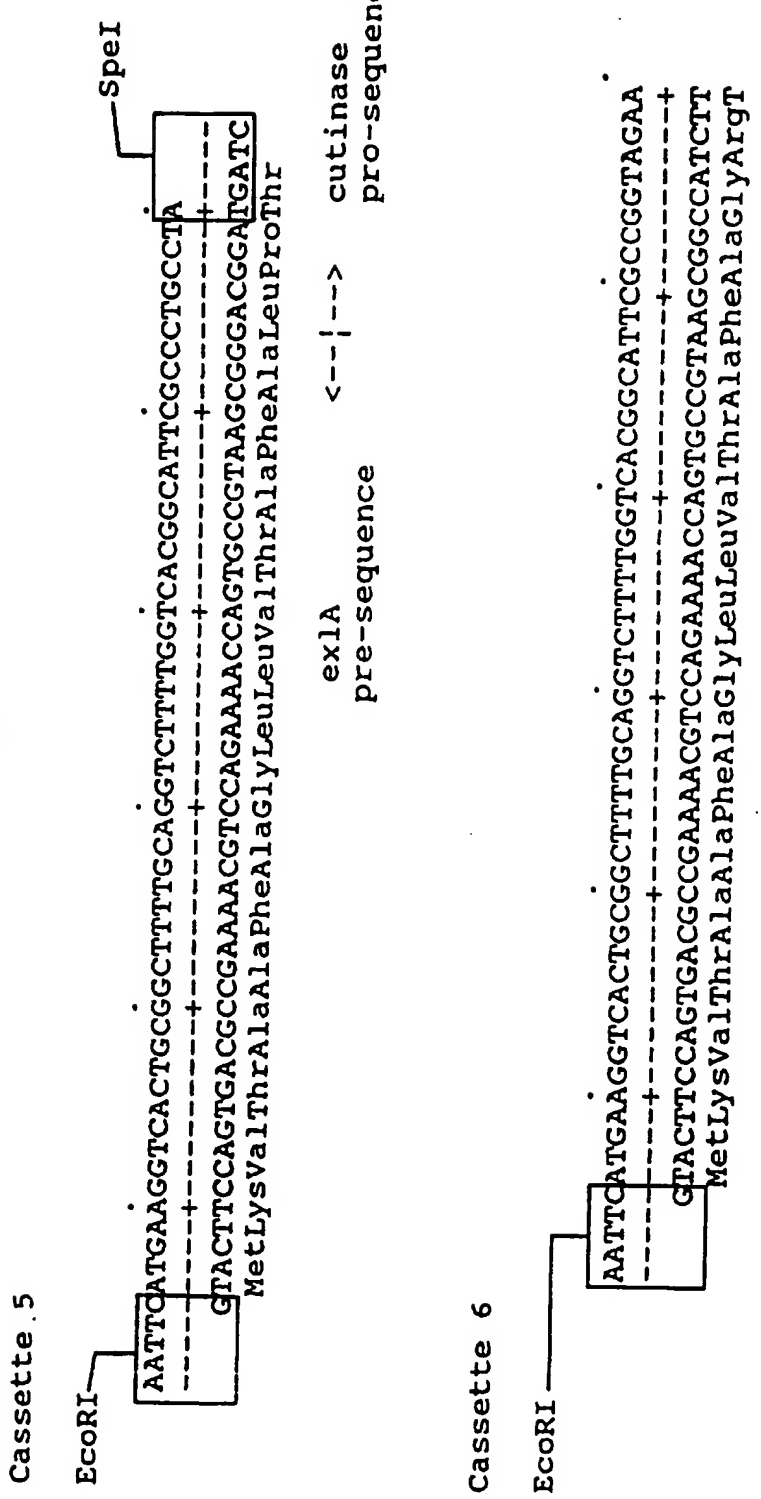

BglII
BamHI
SacI
PvuII
PstI
ScaI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
PstI
HindIII
ori
MB1
PvuII
pUR2740
9212 bps
2 micron
LEU2d
SalI
EcoRI

Fig. 7.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EcoRI
SpeI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCCTGCCTA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGGACGGATGATC
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaLeuProThr
exlA
pre-sequence
<--|-->
cutinase
pro-sequence
Cassette 6
EcoRI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCGGTAGAA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGCCATCTT
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaGlyArgT
exlA
pre-sequence
<--|-->

Fig. 7(Cont).

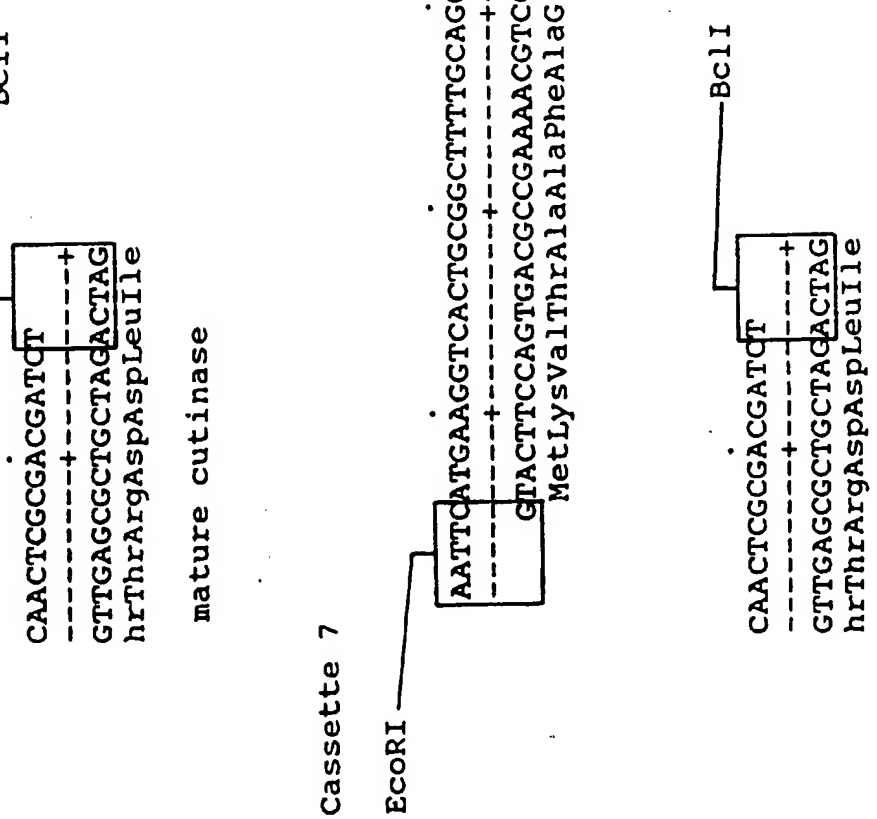

BclI
CAACTCGCGACGATCT
GTTGAGCGCTGCTAGACTAG
hrThrArgAspAspLeuIle
mature cutinase
Cassette 7
EcoRI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCTCAAGAA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGAGTTCTT
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaSerArgT
exlA
pre-sequence
BclI
CAACTCGCGACGATCT
GTTGAGCGCTGCTAGACTAG
hrThrArgAspAspLeuIle
mature cutinase

Fig. 8.

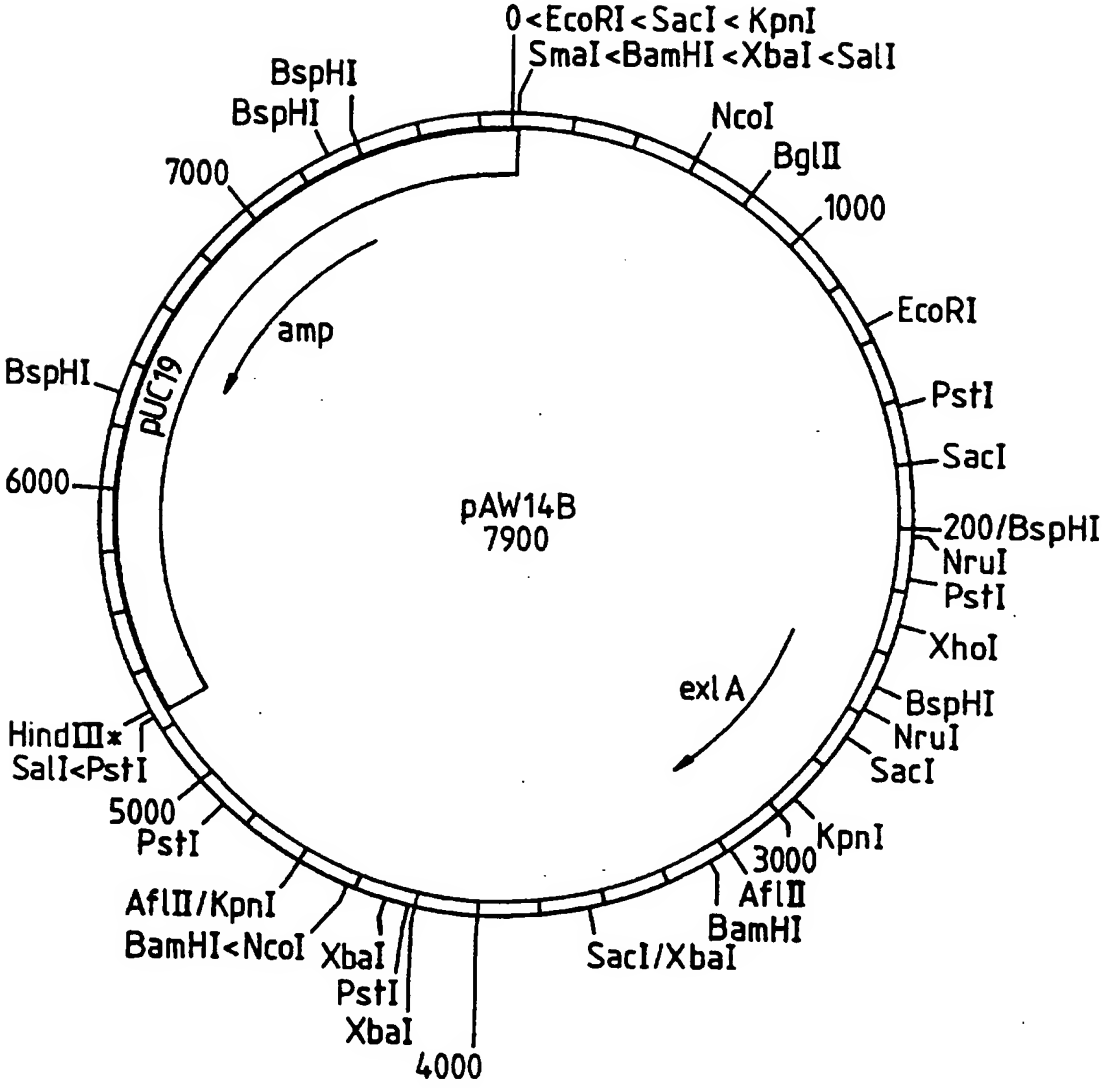
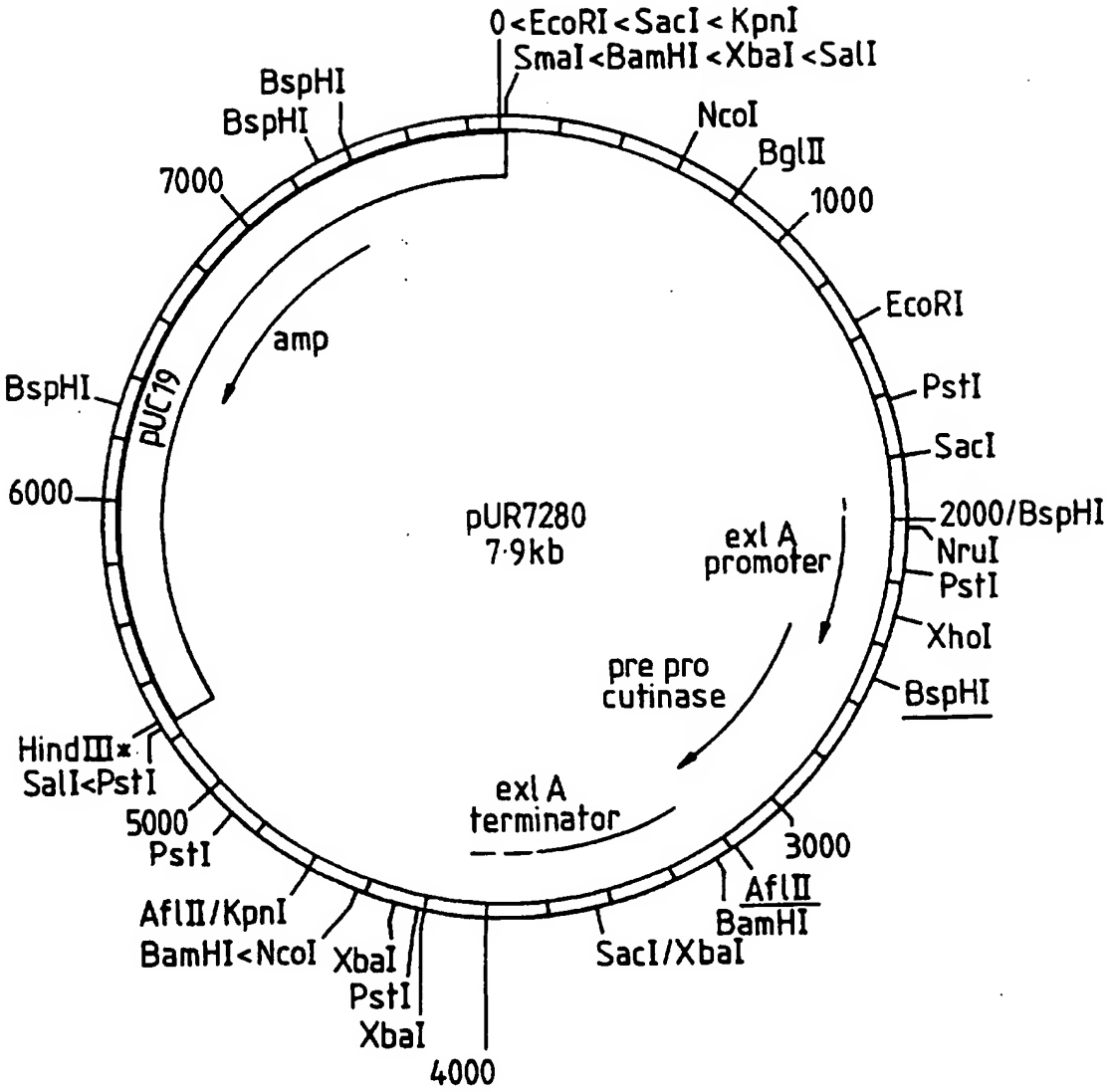

0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
7000
EcoRI
amp
BspHI
pUC19
PstI
SacI
6000
pAW14B
7900
200/BspHI
NruI
PstI
XhoI
exl A
BspHI
NruI
SacI
HindIII*
SalI<PstI
5000
PstI
KpnI
3000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
SacI/XbaI
4000

Fig. 9.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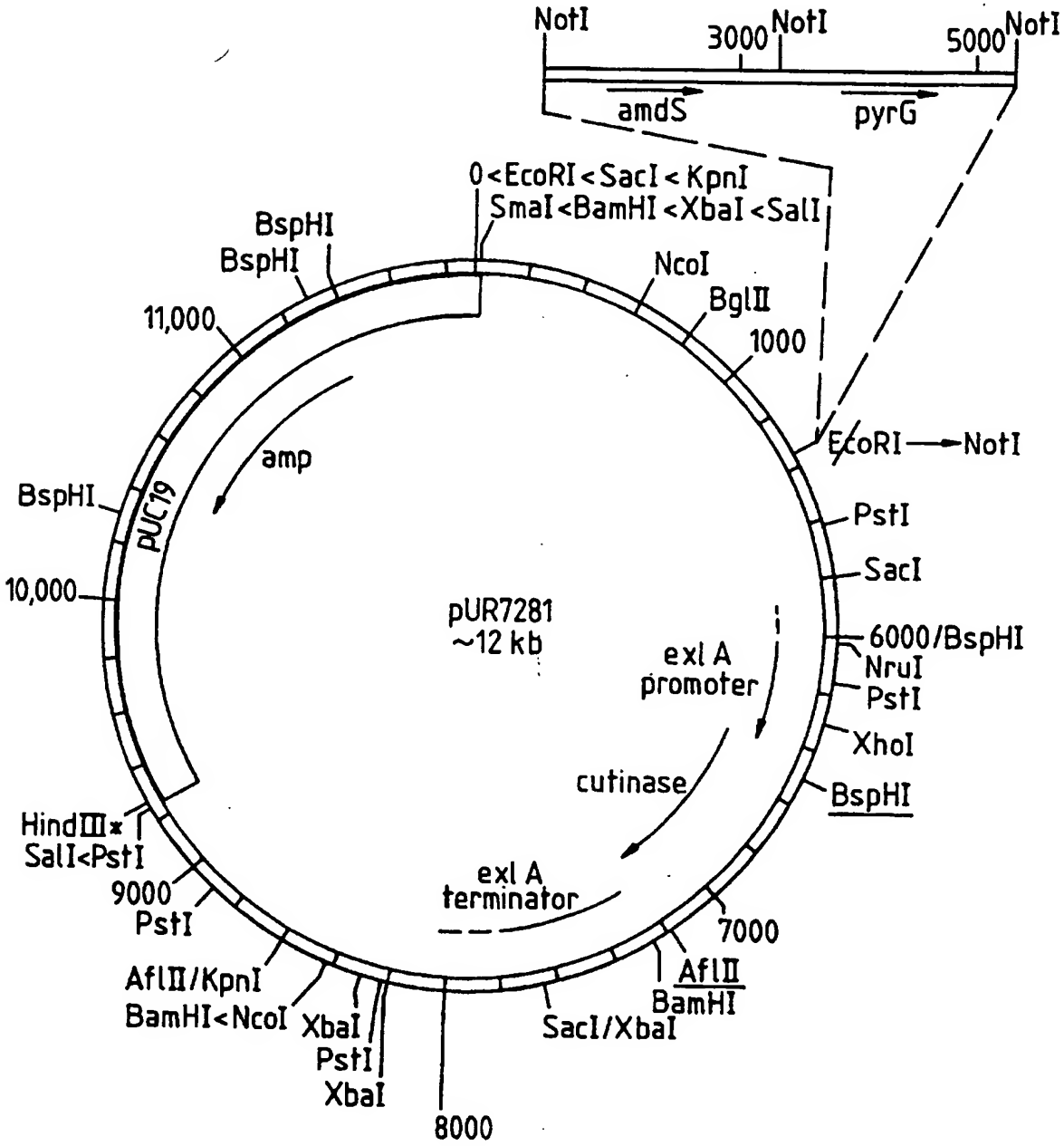
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
7000
EcoRI
amp
BspHI
pUC19
PstI
SacI
6000
2000/BspHI
NruI
PstI
pUR7280
7·9kb
exl A
promoter
XhoI
BspHI
pre pro
cutinase
HindIII*
SalI<PstI
exl A
terminator
5000
PstI
3000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
SacI/XbaI
4000

17/21

Fig.10.


NotI
3000
NotI
5000
NotI
amdS
pyrG
0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
11,000
amp
EcoRI→NotI
BspHI
pUC19
PstI
SacI
10,000
pUR7281
~12 kb
6000/BspHI
NruI
PstI
exl A
promoter
XhoI
cutinase
BspHI
HindIII
SalI<PstI
9000
PstI
exl A
terminator
7000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
SacI/XbaI
8000

18/21

Fig.11.

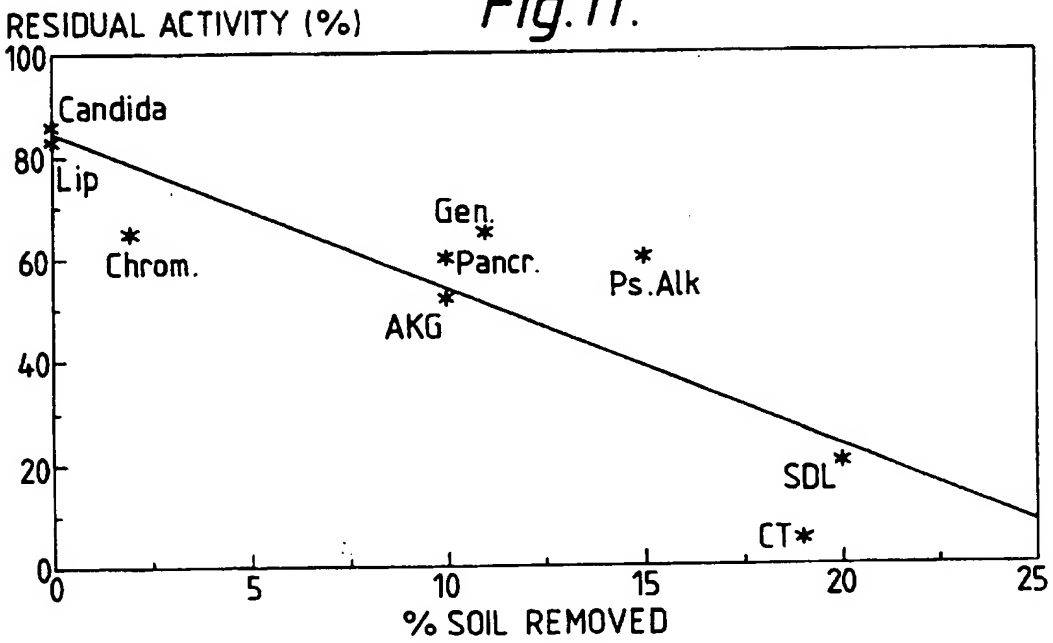


Fig.12.

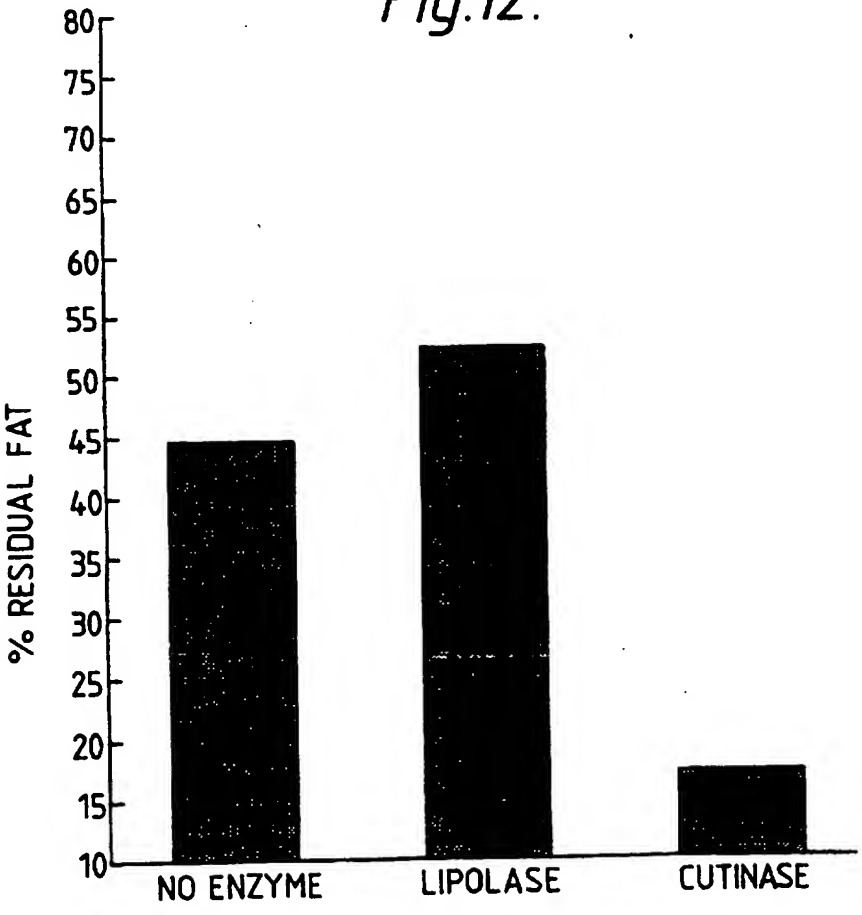


Fig.13.

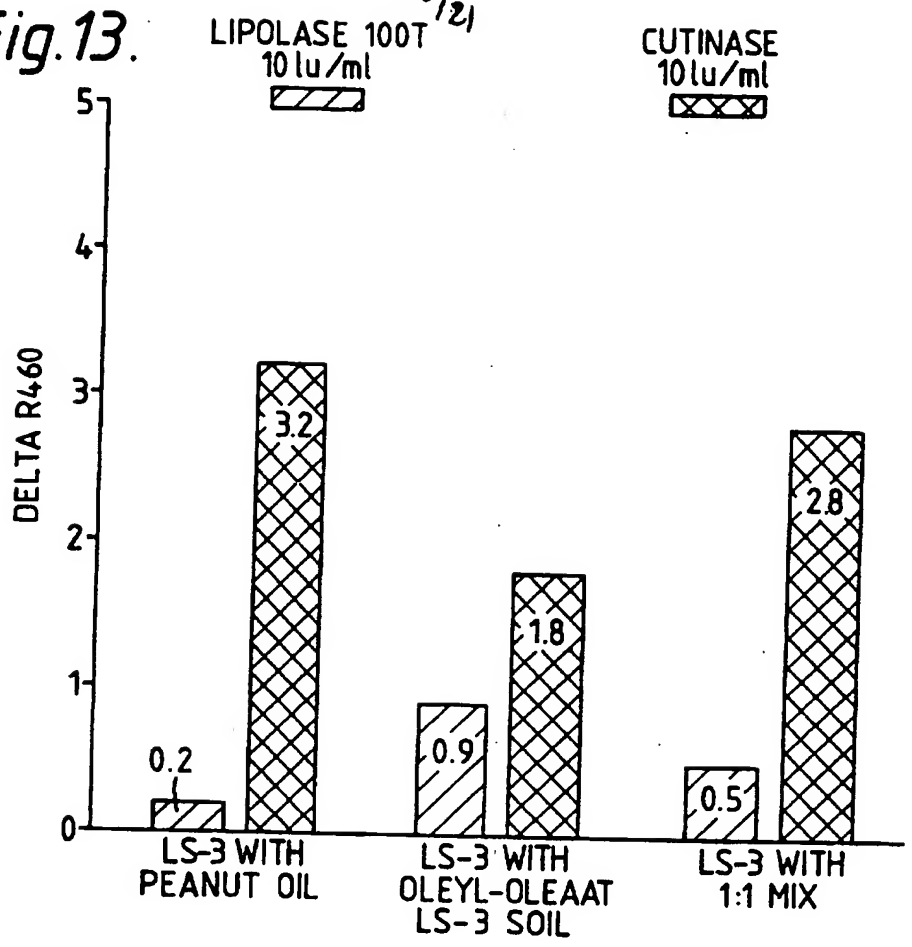


Fig.15.

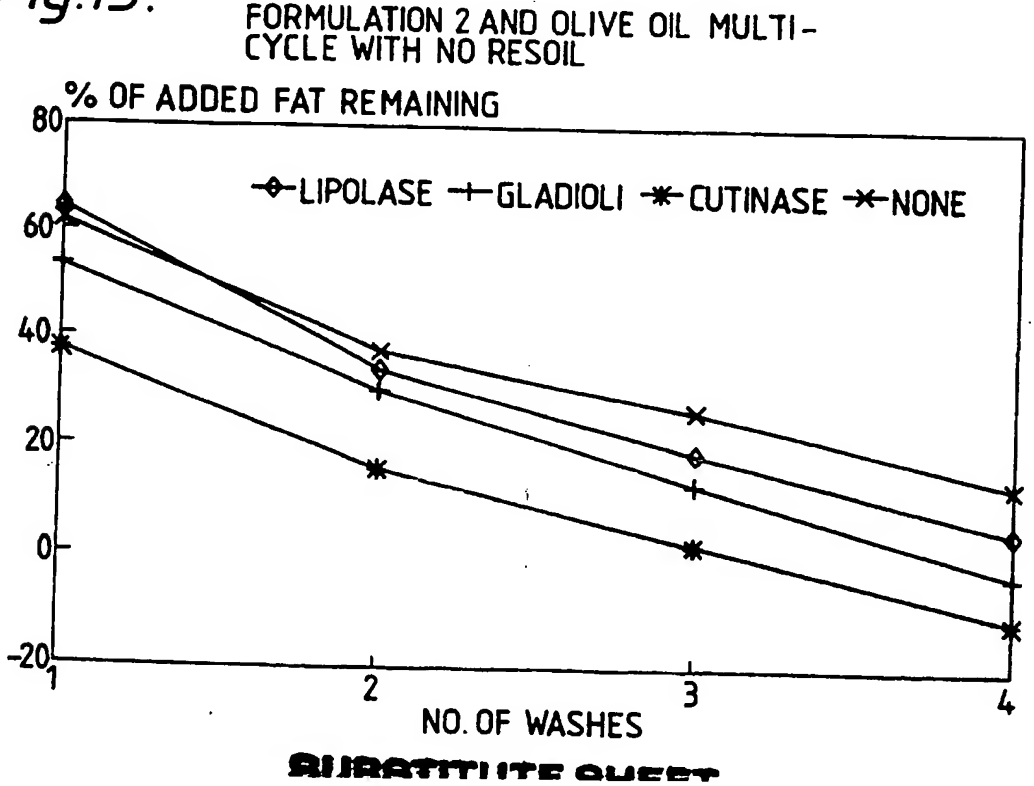


Fig.14A.

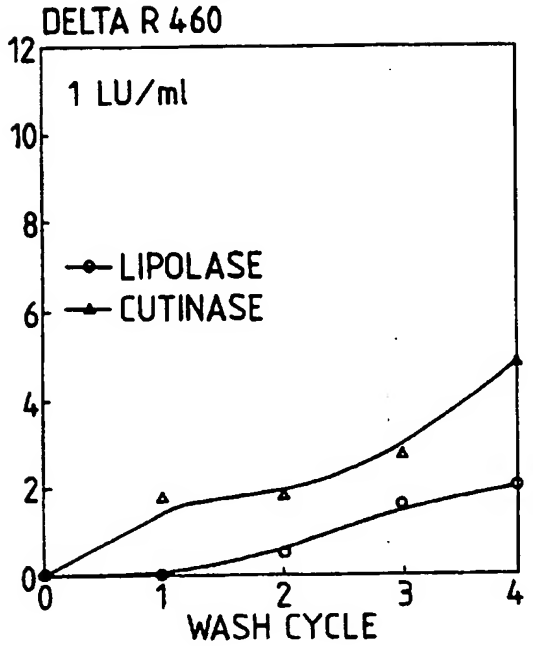


Fig.14B.

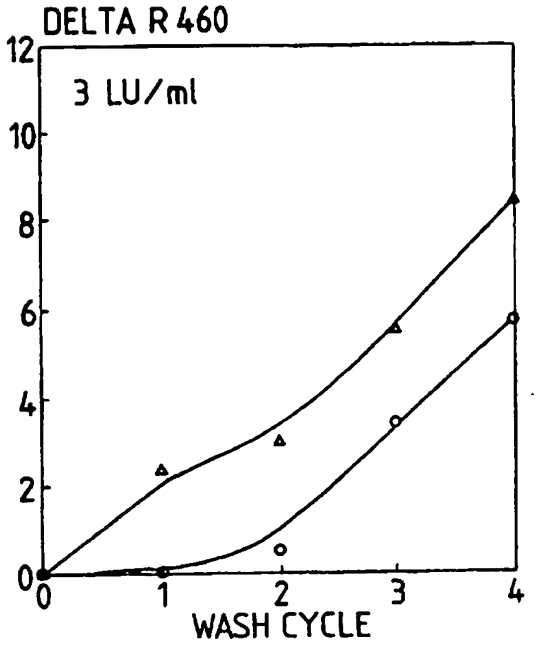


Fig.14C.

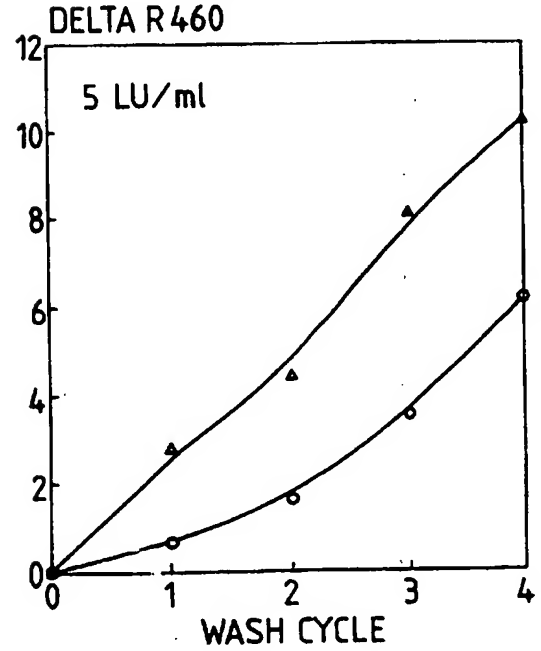


Fig.14D.

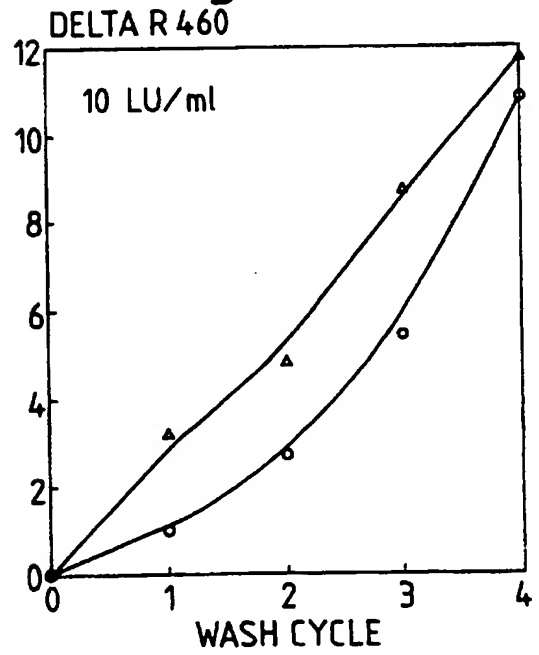


Fig.16. FORMULATION 3 AND OLIVE OIL MULTI-CYCLE WITH NO RESOIL

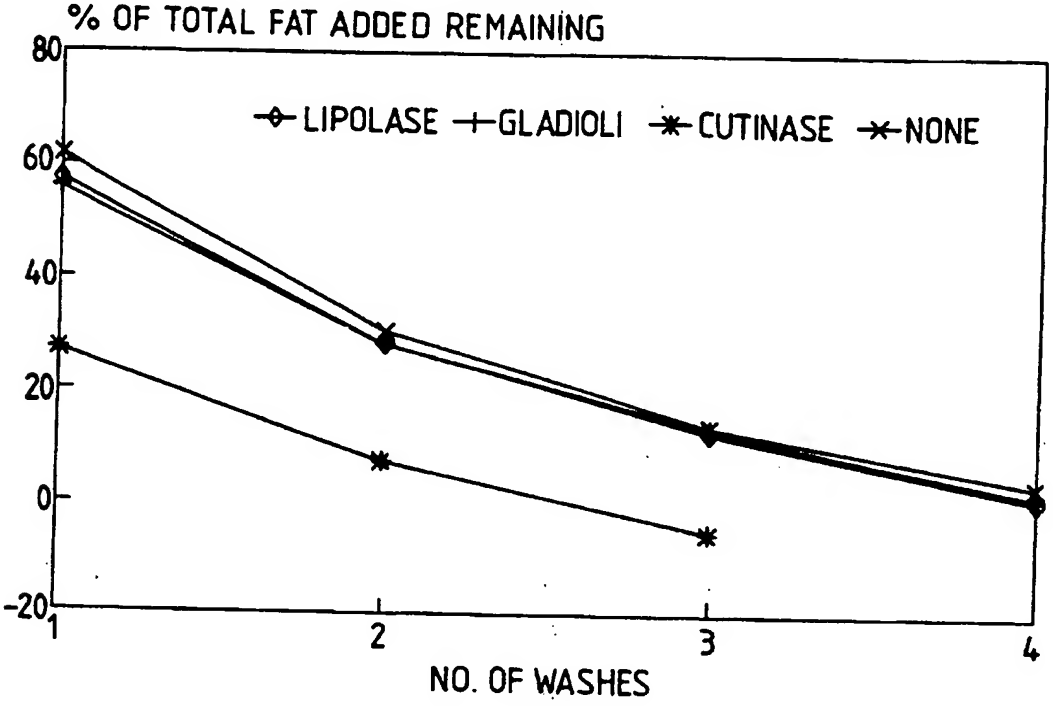
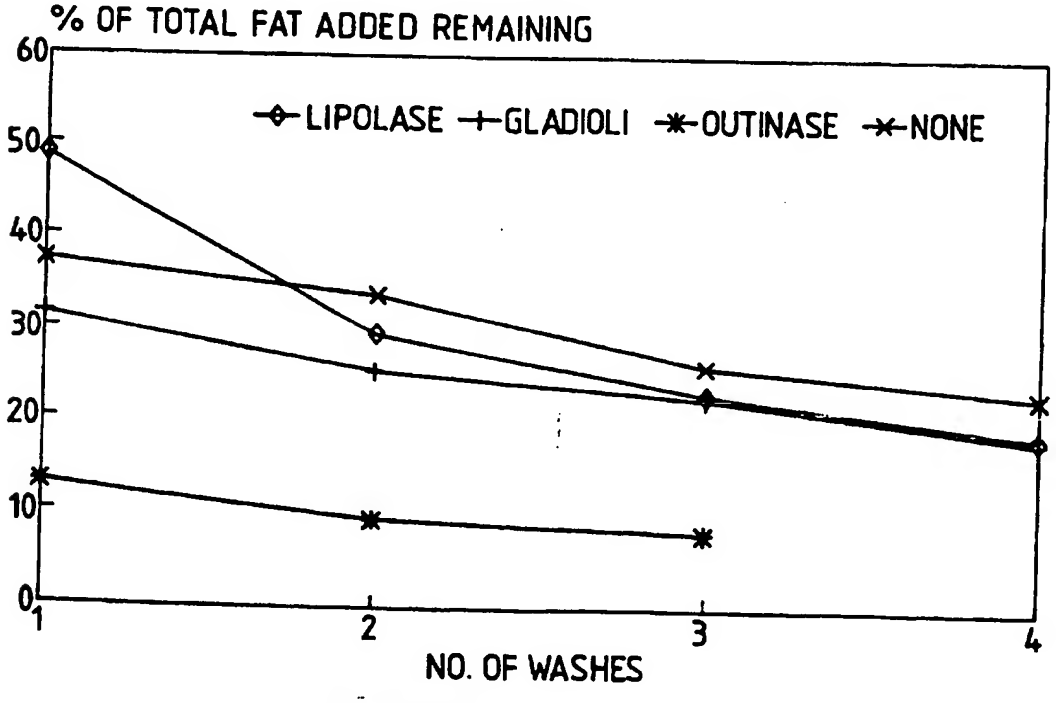


Fig.17. FORMULATION 3 AND OLIVE OIL MULTI-CYCLE WITH RESOIL AFTER EACH WASH



INTERNATIONAL SEARCH REPORT

Int. ...ional Application No
PCT/EP 93/01923

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C11D3/386 C12N9/18 //C12N15/55,C12N9/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C11D C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 09446 (PLANT GENETICS) 23 August 1990 cited in the application see page 17, line 1 - line 8; claims	1-12
A	EP,A,0 399 681 (CLOROX) 28 November 1990 see table II	1
A	WO,A,88 09367 (GENENCOR) 1 December 1988 cited in the application see page 6; claims	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1993	07. 12. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Pfannenstein, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAPPI HOUSEHOLD & PERSONAL PRODUCTS INDUSTRY vol. 28, no. 10 , October 1991 , RAMSEY, US pages 122 - 125 MALMOS, GORMSEN 'A new Lipase for the Detergent Industry' -----	1

1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9009446	23-08-90	NONE		
EP-A-0399681	28-11-90	JP-A-	3088897	15-04-91
		US-A-	5223169	29-06-93
WO-A-8809367	01-12-88	EP-A-	0322429	05-07-89
		US-A-	4981611	01-01-91